PB-217 435

TRACE ELEMENT NEEDS OF FILAMENTOUS MICROORGANISMS

John T. Pfeffer

Kansas University
Lawrence, Kansas

December 1967

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WP-00646

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Center for Research in Engineering Science

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by

John T. Pfeffer, Project Director

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U S Department of Commerce
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Federal Water Pollution Control Administration Research

Grant WP-00646, February 1964 to September 1967

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LITERATURE REVIEW

The literature is replete with reports of sludge bulking as associated with the growth of the filamentous bacterium, Saphaerotilus. Other reports associate bulking with bound water in the sludge. Since this study was concerned with the relationship between fungal growth and sludge bulking, the review will be confined to this literature.

Some of the earliest work was done by Smit (1) in 1934. He published photomicrographs of a variety of filamentous microorganisms found in bulking sludge. He preferred not to specifically name the organisms, but did suggest that three of the four organisms involved were fungi. In 1940, Lackey and Wattie (2) concluded that sludge bulking can be associated with several organisms, but usually the predominant organism is the filamentous bacterium Sphaerotilus natans Kutzuig. They point out the extreme variability in the growth characteristics of the organisms and the difficulty in distinguishing the several varieties.

Jones (3) studied the effect of temperature and oxygen tension on the growth of what was thought to be Sphaerotilus. As the work progressed, the organism was identified as the fungus, Geotrichum. The same problem of identification was experienced by Pipes and Jones (4). These two organisms have such similar morphological characteristics that identification is extremely difficult. This may in part, be responsible for the relative lack of reports of fungi as the causitive organisms in sludge bulking.

Ruchhoft and Kachmar (5) showed experimentally that bulking was a response of the sludge organisms to a sudden disturbance in the biological equilibrium; especially to three primary factors including sludge, nutrients, and rate of oxygen supply. It was found that in certain cases of bulking the disturbance producing it was accompanied by a great increase in Sphaerotilus, although other organisms present in activated sludge could cause bulking under other disturbed conditions.

Hawkes (6) found Geotrichum in the activated sludge plant at Birmingham, England. He reported this organism to be the causitive agent in sludge bulking problems experienced at this plant.

Fungi are common in polluted waters. There are numerous reports in the literature on the occurrence of various species of fungi in trickling filters, streams, and activated sludge. Cooke (7) presents an extensive literature survey of the fungi in polluted waters. He lists the following

fungi as being important in polluted waters; i.e. Leptomitus, Geotrichum, Sepedonium, and Fusarium. Other genera such as Oospora and Oidium have been found in polluted waters. It is possible to find a large number of species of fungi in waste waters. However, it is only when the chemical and physical environments are such that these fungi become the predominant growth that they are of any consequence.

1. Nutritional Requirements of Fungi

Most of the nutritional studies involving fungi have been made on organisms used for commercial applications or those fungi important as plant and animal pathogens. Textbooks of fungi physiology (8,9) describe the basic vitamin requirements and the types of sources of carbon and nitrogen which are metabolized by various fungi. In addition, limited information on fungi nutrition is available in texts on industrial microbiology.

Cooke (10) studied the nutrition of nine fungi common to waste water. These fungi included Fusarium and Geotrichum. Table 1 lists the growth response of these two genera as given by Cooke. Growth of these organisms was good on all simple sugars tested. There was some variation in the nitrogen source with Geotrichum requiring organic nitrogen, or more specifically the lack of nitrate. Vitamin requirements were variable, but in general, good growth was obtained on all vitamins tested.

Most fungi found in polluted waters are capable of metabolizing all of the carbohydrates. There is a general preference for organic nitrogen contrasted to ammonia and nitrate nitrogen. External vitamin sources are required by a few fungi, but most of these organisms are capable of producing their own vitamins.

The requirements for trace or micronutrients are much more varied than the major nutrients. Texts on the physiology of fungi (8,9) suggest that fungi need about 17 different elements so supply their nutritional requirements. These elements may be utilized in the form of specific compounds, as ions, and as free elements. Some of the essential elements are required by all fungi, where as certain elements are not required by some species. In general, the elemental requirements of the fungi are essentially the same for bacteria, algae and larger plants and animals. There can be, however, striking differences in the essential element requirements of any given species of organisms.

TABLE 1

GROWTH OF FUNGI ON VARIOUS VITAMINS, NITROGEN AND CARBON SOURCES

Nutrients	F. aquaeductuum	F. oxysporum	G. candidum
Vitamins			
Thiamine	+	4	4
Biotin	4	4	4
Thiamine + Biotin	3	4	4
Thiamine, Biotin, Inositol, Pyridoxine	3	3	4
Nitrogen			
l-Asparagine	4	4	4
$(NH_4)_2\ SO_4$	2	4	3
$NH_4\ NO_3$	2	3	+
KNO_3	3	4	+
$Na\ NO_3$	3	3	+
Urea	3	3	4
Carbon			
Xylose	2	2	3
Glucose	4	4	4
Mannose	4	3	4
Fructose	2	4	4
Sucrose	4	4	2
Lactose	1	4	+
Soluble Starch	4	4	+

+ - Visible Growth, 1 - Poor Growth, 2 - Moderate Growth

3 - Good Growth, 4 - Excellent and Dense Growth

The exact biological purpose of various elements is varied. Thatcher (11) has attempted to classify the essential elements into groups according to their role; i.e. structural, functional and those utilized in the transfer of energy. This classification has some validity and may serve to fix attention upon the more salient biological features of an element.

The problem of determining which elements are essential for fungi has been approached from the standpoint of ultimate analysis of mycelium and spores. If certain elements such as potassium and magnesium are always found in all samples analyzed, irrespective of the substrate upon which these fungi were cultivated, it may be concluded with a high degree of probability that these elements are essential for the fungi. Buchanan and Fulmer (12) and Foster (13) collected analytical results of ultimate analysis of mycelium and spores.

2. Essential Macroelements

Potassium. This element is essential for all organisms, so far as is known. There is a tremendous amount of information on the specific effects of potassium on green plants and animals, but such data are not common for the fungi. Steinberg (14) studied the quantitative relationship between the amount of potassium in the medium and the weight of mycelium produced by Aspergillus niger. This work was done with extraordinary care using a highly purified optimal media with varying amounts of potassium. The optimum potassium concentration in the medium was 150 mg/l. The relative amount of mycelium formed increased as the potassium content of the medium decreased. Molliard (15) noted that a low potassium content in the medium resulted in increased synthesis of oxalic acid by A. niger. Rippel and Behr (16) found that the chemical composition of A. niger mycelium varies, depending upon the amount of potassium in the medium.

Magnesium. This element is one of the alkaline earth group. It is essential for green plants and animals as well as for fungi and bacteria. Aspergillus niger has been more carefully investigated with respect to the effects of magnesium than any other fungus. Steinberg (17), and Lavalloy and Laborey (18) demonstrated that within certain limits of concentration, the amount of growth of A. niger is proportional to the concentration of magnesium in the medium. Painter (19) studied various aspects of nutrition of four fungi which commonly inhabit trickling filters, but he did not

indicate the magnesium requirements of these organisms. Steinberg (20) in a study of the relationship of carbon nutrition to trace elements reported that magnesium was necessary for the growth of A. niger irregardless of the carbon source.

3. Essential Microelements

These elements have been called heavy metal nutrients, trace elements, micronutrients and minor elements. The literature on this subject is extensive and often conflicting. Steinberg (21), Foster (22), and Perlman (23) have reviewed this subject. Earlier investigators considered trace elements to be stimulatory, rather than essential. This view is no longer held.

Calcium. This element was one of the first to be recognized as essential for green plants and animals. Young and Bennett (24) reported that Rizoctonia solani did not exhibit any growth in the absence of calcium. Steinberg (25) studied the calcium requirement of seven fungi and reported that the quantity required for maxiumu yield ranges from 2 to 6 mg/l. Lindeberg (26) found a synergistic effect between manganese and calcium on the growth of various species of Marasmius.

Manganese. The classification of this element as essential rests upon the experimental findings that omission of this element from media resulted in a decreased yield. The multiplicity of examples strengthens the validity of this conclusion, although most researchers have confined the attention to a relatively few species. The results of a study by Robbins and Hervey (27) with Pythiomorpha gonapodyoides indicate that studies on trace nutrient needs of fungi may be more rewarding if species other than A. niger are used. McHargue and Calfee (28) noted that the growth of A. flavus, Rizopus nigricons, and Saccharomyced cerevisiae increased in the presence of added manganese. Steinberg (29) showed that omission of manganese from a balanced medium resulted in a decrease in yield of A. niger from 1,084.8 to 356.6 mg. Sporulation was absent when manganese was omitted.

The list of metallic ions known to be essential to fungi has increased over the years. The list now includes potassium, magnesium, iron, zinc, copper, calcium, manganese, molybdenum. Others will probably be added as cultural methods become more refined and more species are studied. It is unfortunate that only a few fungi have been investigated throughly with

respect to mineral nutrition. In stating that the above elements are essential, the reservation must be made that they are essential for some fungi under certain conditions. While it may be assumed that all fungi require the same essential elements, experimental evidence is lacking for most species.

The widespread use of A. niger as a test organism in microelement studies has had the advantage that the work in many laboratories may be compared. The careful and continued studies of Steinberg are especially valuable. The exclusive use of A. niger has also had its disadvantages in that little is known of the micronutrient requirements of other species.

Perlman (30), after reviewing some 175 reports of the effects of trace elements in the physiology of fungi, concluded that "Until some effort is made to standardize such techniques as growing inoculum, sporulation, stock cultures and preparation of media it is inadvisable to draw any general conclusions regarding carbohydrate metabolism by fungi or the effects of metallic ions on the metabolism of fungi.

4. Availability of Microelements

The presence of the various metal ions in a substrate does not necessarily mean that they are available to the microorganisms. These ions must be in true solution for the organism to be able to use them. There must not be any interaction between the metal cations and various anions present in a biological substrate. This is especially important when considering a substrate such as domestic or industrial waste water.

An examination of the various cations considered in microorganism nutrition will provide an indication of the ions that are likely to be limiting as a result of chemical interactions. Cations such as potassium, sodium, calcium, and magnesium will not precipitate at pH levels normally encountered in biological systems. When operating at pH levels 8.5 or greater, calcium phosphate precipitates may result. This pH is generally never reached in most biological water treatment systems.

This problem really manifests itself with iron, zinc, cobalt and manganese. The solubility of these cations is limited by several precipitation reactions. The solubility is further reduced when some of these cations are in the oxidized state, which occurs in aerobic biological systems.

The solubility of iron (ferric) is controlled by several equilibrium systems. This is best shown by the following figures. Figure 1, after Black (31), shows the solubility of iron (ferric) in an aqueous environment void of carbonates, phosphates and sulfur. There are four ion species formed which control the solubility. The minumum solubility is about 10^{-9} moles/l at a pH of 9.0. At pH of 7.0, the solubility is about 10^{-7} moles/l or about 0.05 mg/l of iron. The solubility problem is complicated by the presence of carbonate, sulfur and phosphate plus the oxidation-reduction potential of the system. Figure 2, after Hem (32), is an Eh-pH plot for iron solubility in system containing 10^{-4} moles/l of sulfur and 10^{-3} moles/l of carbonate. At a pH of 7.0, the solubility of iron is 10^{-4} moles/l with an Eh between -0.15 to +0.08 volts. If the system is highly oxidized, the Eh values increase with a resulting decrease in the iron solubility.

The presence of phosphate in the system will drastically reduce the solubility of the iron. The equilibrium system among the various species of phosphate ion will keep the concentration of PO_4^{-3} ion at very low levels. Figure 3 shows the relationship between the phosphate ion species and pH. This curve was constructed from the ionization constants given by Baard (33). From this figure, it can be seen that the PO_4^{-3} concentration is very low at a pH below 9.0. Table 2 lists the concentration of PO_4^{-3} ion in a 10^{-4} molar phosphate system. With these extremely low concentrations of PO_4^{-3} most metals would be soluble. However, the K_S for $FePO_4$ is given as 10^{-22}. With this low value, iron will be precipitated by PO_4^{-3} ion even at low pH levels. Table 3 lists the solubility of iron (111) in a 10^{-4} molar phosphate system. It can be seen that iron will be in the concentrations range of 10^{-11} moles/l even at pH levels of 6.0. Considering Figure 1 and Table 3, the solubility of iron would be at extremely low levels in a system operating at pH levels of 7 to 8. Analysis of waste treatment plants show iron to be in solution at levels of at least a few tenths of a mg/l.

The iron is maintained in solution by complex formation with constituents in the waste water. Stumm (34) suggests that very little complex formation occurs in waters containing dilute concentrations of inorganic salts. The OH^- ions form complexes, but these are the only stable complex formations found. The distribution of various soluble and insoluble forms can be described quite adequately in terms of mass-law equilibrium relationships, especially if the equilibrium constants have been corrected for non-ideal behavior.

FIGURE 1

SOLUBILITY CURVE FOR FERRIC HYDROXIDE

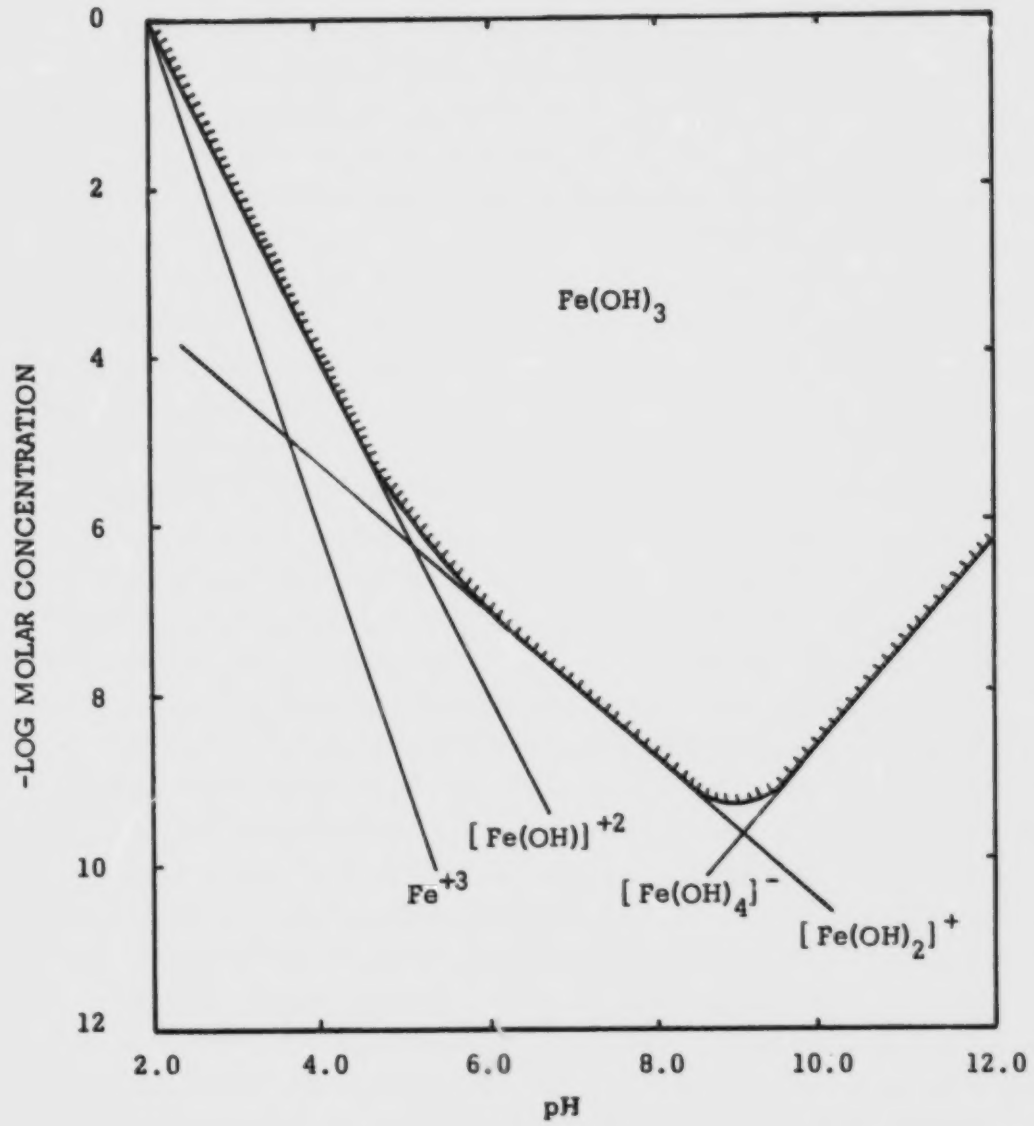


FIGURE 2

SOLUBILITY OF IRON IN RELATION TO pH AND Eh AT 25°C AND 1 ATM. TOTAL DISSOLVED SULFUR, 10^{-4}M; BICARBONATE SPECIES, 10^{-3}M.

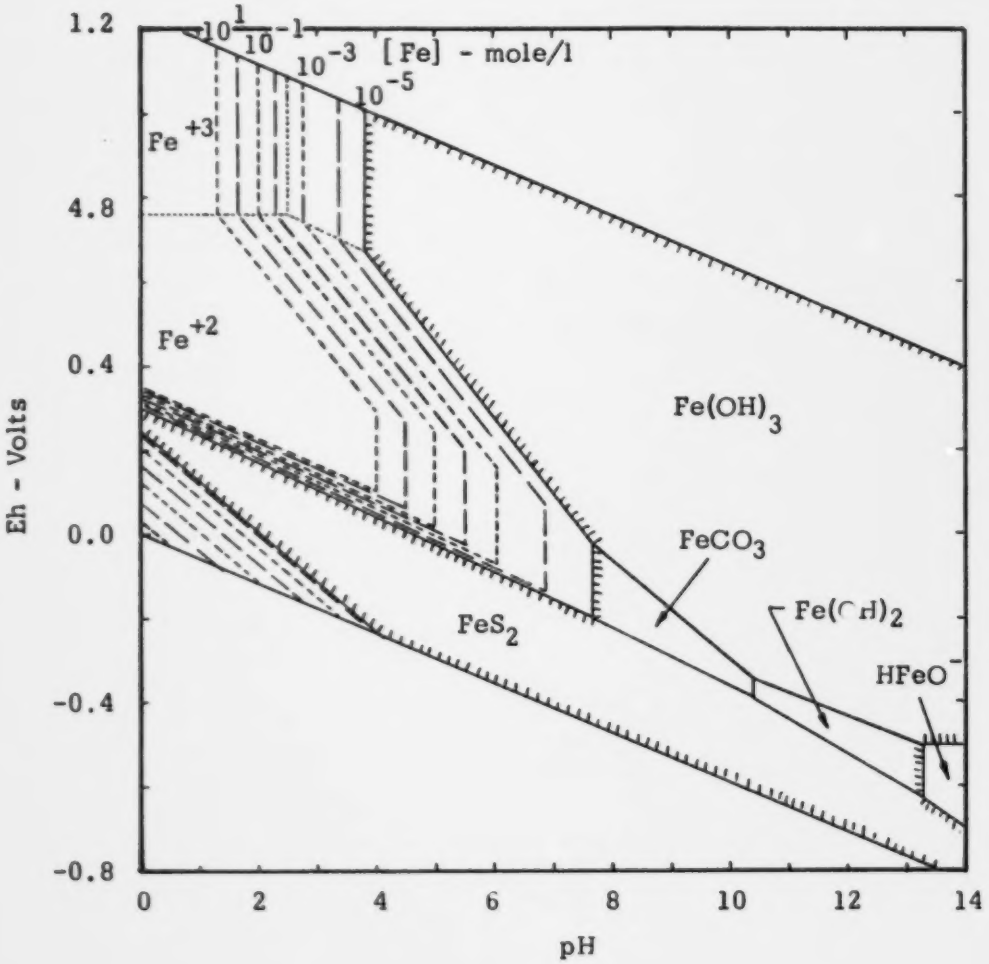


FIGURE 3

PHOSPHATE ION SPECIES DISTRIBUTION
AT VARIOUS pH LEVELS

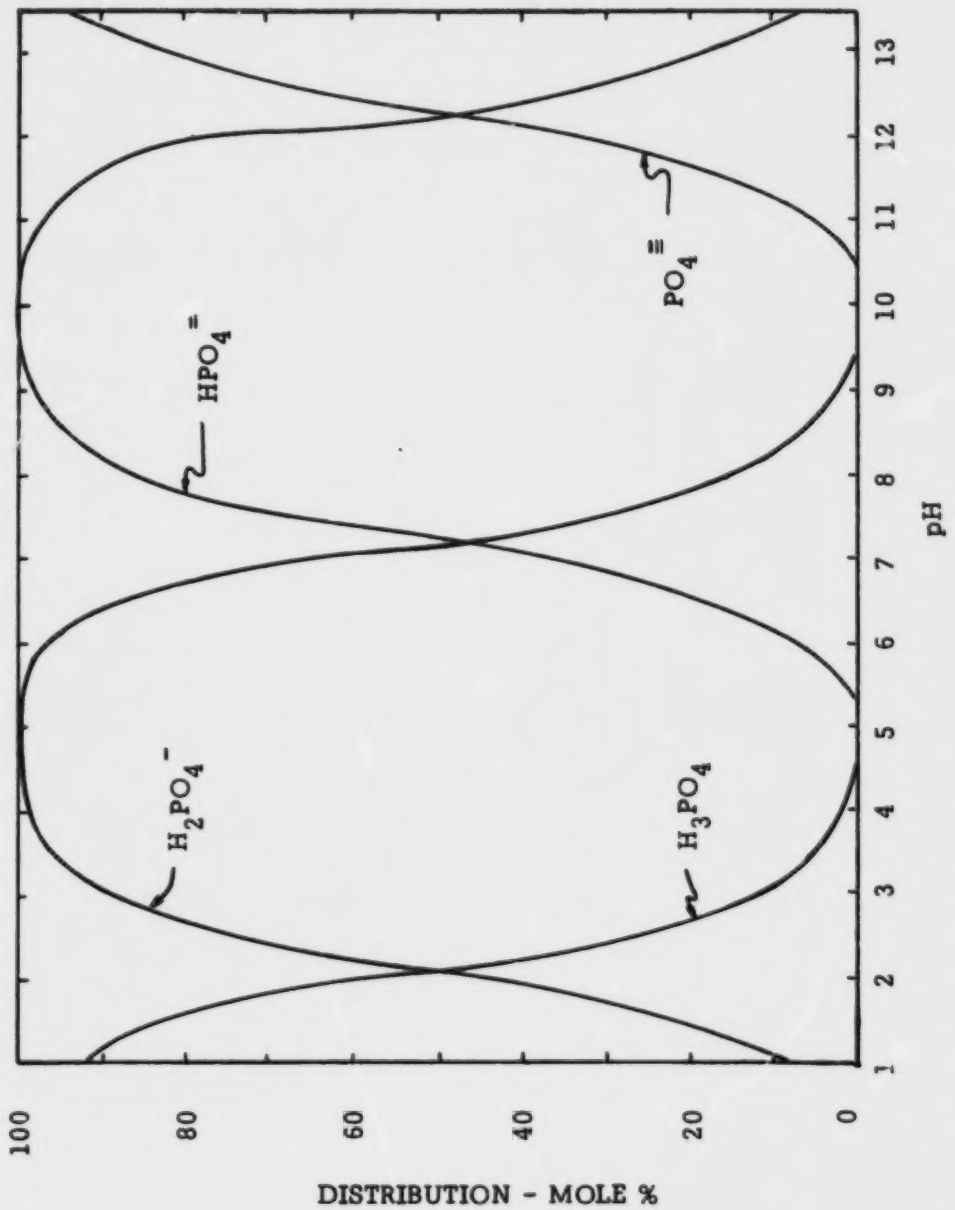


TABLE 2

PO_4^{-3} CONCENTRATION IN A 10^{-4} MOLAR PHOSPHATE SYSTEM

pH	PO_4^{-3} (moles/l)
4.0	3.3×10^{-16}
5.0	3.3×10^{-14}
6.0	3.3×10^{-12}
7.0	1.82×10^{-10}
8.0	4.1×10^{-9}
9.0	4.8×10^{-8}

TABLE 3

SOLUBILITY OF IRON(III) IN A 10^{-4} MOLAR PHOSPHATE SYSTEM

pH	Fe^{+3}(moles/l)
4.0	3×10^{-7}
5.0	3×10^{-9}
6.0	3×10^{-11}
7.0	5.5×10^{-13}
8.0	2.4×10^{-14}
9.0	2.1×10^{-15}

A chelating effect can be observed with complex formation of metal ions with organic compounds. Amines and other nitrogenous compounds play an important role in complex formation of several metal ions. Yatsimirkii and Vasilev (35) have an extensive listing of organic complexes. Table 4 lists several forms of iron complexes with the associated instability

TABLE 4

IRON COMPLEXES (From Yatsimirskii and Vasilev (35))

Complex	Instability Constant
Dipyridylamine	
$Fe\ Dip^{++}$	6.1×10^{-5}
$Fe(Dip)_2^{++}$	6.1×10^{-9}
$Fe(Dip)_3^{++}$	2.6×10^{-18}
Diethylenetriamine	
Fe Deta++	5.9×10^{-7}
$Fe(Deta)_2^{++}$	4.4×10^{-11}
Ethylenediamine	
$Fe\ En^{++}$	5.25×10^{-5}
$Fe(En)_2^{++}$	2.95×10^{-8}
$Fe(En)_3^{++}$	3.01×10^{-10}
Alanine	
$Fe(Alan)_2$	5.0×10^{-8}
Asparagine	
$Fe(Asp)_2$	3.16×10^{-7}
Glycine	
$Fe(Gl)_2$	1.6×10^{-8}
Acetyacetone	
$Fe\ Acac^{++}$	1.6×10^{-10}
$Fe(Acac)_2^{+}$	1.6×10^{-19}
$Fe(Acac)_3$	1.6×10^{-27}

constants. Many of these complexes are very stable as evidenced by the magnitude of the constants. This table is by no means complete, but it does represent some of the organics that may be responsible for the complex formation in wastewater.

Stumm (34) suggests that pH markedly influences the degree of complex formation. At low pH, H^+ competes successfully with the ligand for the coordinative positions on the metal ion. Furthermore, at low and high pH, mixed hydrogen-metal and hydroxide-ligand complexes can be formed. Because of the competing influence of H^+ or OH^-, the complexing effect can not be estimated solely from the instability constants.

Iron is the metal that is most likely to be unavailable as a result of chemical precipitation. However, manganese solubility is limited by the formation of several species of ions. Figure 4, after Morgan (36), shows the solubility of Mn^{++} in water containing the carbonate species. At pH levels of 7.0, the solubility of manganese is about 10^{-4} moles/l. Oxidation of the Mn^{++} to Mn^{+4} will change the solubility substantially. This can be seen in Figure 5 as presented by Morgan (36). As the Eh becomes positive the solubility decreases as a result of the formation of the insoluble oxidized manganese compounds. Figure 5 is for water containing only the carbonate species. The additions of organics such as found in waste water will allow for the formation of organic complexes. Manganese exhibits many properties similar to iron. Examination of the literature shows complex formations similar to the iron complexes in Table 4. The constants are, in general, somewhat greater. There can be no question though as to the importance of the complex formation in maintaining the metals in solution and available to the microorganisms.

Curves similar to those presented for iron and manganese could be constructed for the various metal ions of concern. There would be certain areas where solubilities are limiting the availability of the cations for use by the microorganisms. In general, one can expect organic complex formation to keep these ions in solution when dealing with a waste water such as domestic sewage. However, these organic complexes may not be available in certain industrial wastes and also in chemical media used for laboratory studies. The precipitation of certain cations may then result in inhibition of bacterial growth. If this particular cation is not essential to fungal growth, the fungi will then predominate in the biological process with the problem of bulking sludge resulting.

FIGURE 4

SOLUBILITY OF Mn(II) IN WATER CONTAINING
2×10^{-3} M TOTAL CARBONATE SPECIES

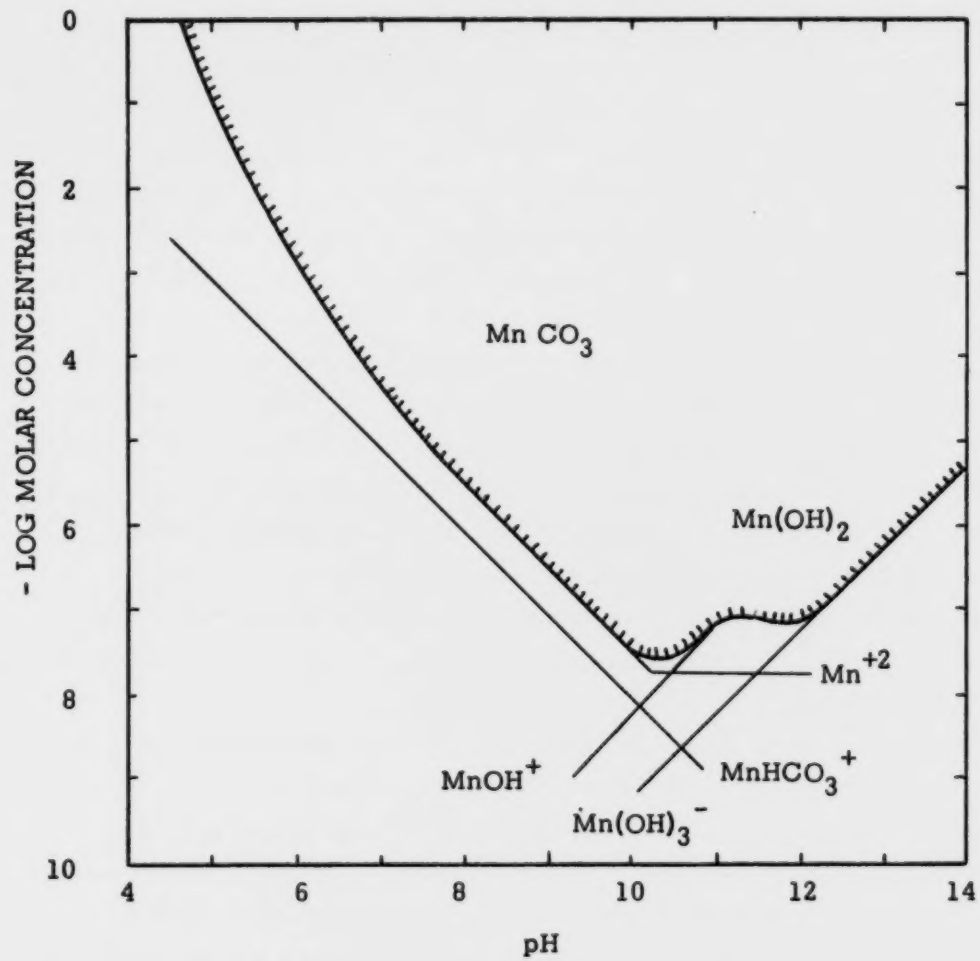
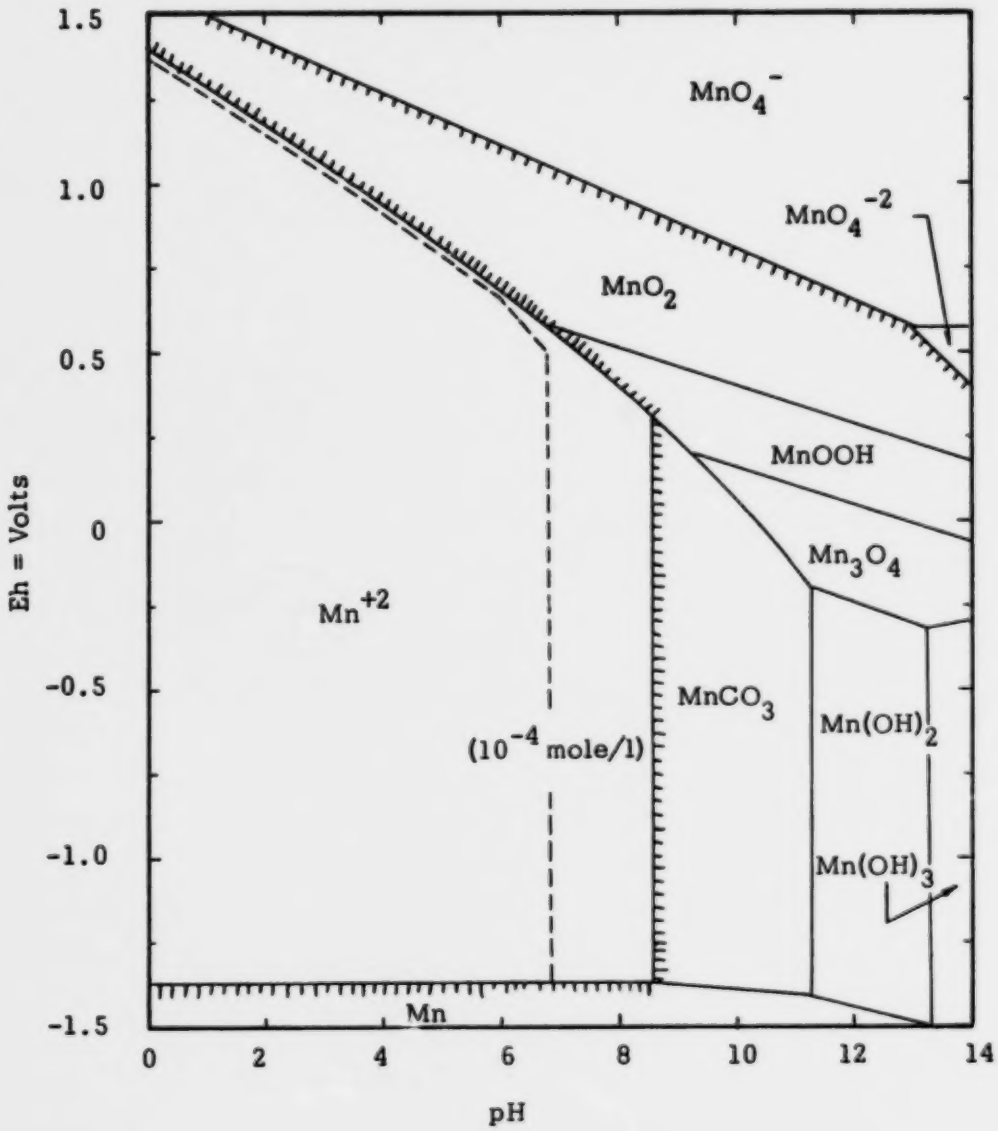


FIGURE 5

MANGANESE POTENTIAL DIAGRAM
FOR Mn, O_2, CO_2, H_2O, 25°C, 10^{-6}M



Consideration should be given to the possibility that certain of these metals may not have been in the waste water originally. In excess lime treatment of water, very high removal of any iron and manganese can be expected. These metals may be eliminated as a result. Only through the addition of waste material containing iron can this metal be added to the waste water.

EXPERIMENTAL PROCEDURE

This study was conducted in two phases. Phase 1 was a study of the growth characteristics of filamentous fungi in pure culture. A chemically defined substrate was used and the growth response to the absence of various trace metals was evaluated. Phase 2 consisted of a mixed culture study of the growth of fungi and bacteria in a chemically defined substrate. The growth response of the microorganisms to the absence of various trace metals was ascertained by using various combinations of metal ions in the substrate.

1. Isolation of Fungi - Phase 1

A sample of raw sewage was obtained from the Lawrence sewage treatment plant. The sewage was placed into flasks and aerated for 48 hours on a gyrotory shaker operating at about 200 cycles per minute. This rate provided thorough agitation. The sample was then allowed to stand quiescently for 30 minutes. After this time, 200 ml of supernatant were withdrawn and replaced with 200 ml of synthetic substrate. The composition of this substrate is given in Table 5. The concentrations used in this isolation step was one-fifth that given in Table 5. To encourage the growth of fungi over bacteria, various trace metals were eliminated from the media.

After the initial feeding, 200 ml of substrate were added to the flasks daily. This addition followed withdrawal of 200 ml of supernatant after a 30 minute setting period. This procedure was continued until the growth was predominately fungi. This was determined by the appearance of the floc and by microscopic examination. The cultures were then streaked onto low pH mycological agar for isolation of fungal colonies. The single colonies were transferred to regular mycological agar. These isolates were maintained in this agar and after growth was established, the cultures were stored under refrigeration. The cultures were periodically replated to maintain an active population of microorganisms.

Two predominant genera were identified. These were Fusarium and Geotrichum. Since these organisms were reported in the literature as frequent isolates from waste water, they were selected as the organisms for study. These cultures were easily maintained and the only problem was with atmospheric contamination. Frequent inspections were made of the colony growth

and when contamination was observed, the original organisms were transferred to a new plate to maintain the culture pure. Aspergillus was a very frequent contaminant and was first isolated from the plates in which the sewage samples were streaked. Since this organism is known to be an air contaminant, it was excluded as a test organism.

2. Synthetic Substrate - Phase 1.

The substrate used for the isolation of the fungi and in the early stages of this study is given in Table 5. This substrate is similar to the carbon nutrition medium proposed by Waksman (37). This substrate was very difficult to prepare without obtaining immediate precipitation of the various ions in the substrate. Several techniques for preparing the media were tried, but were unsuccessful as a result of the chemical precipitation of some of the cations. Analysis of the medium immediately after preparation indicated an interaction between the calcium, iron, and zinc; and the phosphate ions. There was also hydrolysis of iron occurring. This was not to be ascertained analytically because of the low iron concentration in the media. It is doubtful that this hydrolysis was a significant mechanism in a media containing a high concentration of phosphate ions.

The following technique was found to be satisfactory in preparing the media without immediate precipitation of phosphate salts. All of the constituents of the media except iron, zinc and phosphate were added to the demineralized water.

TABLE 5

Substrate Composition -Pure Culture Studies (Phosphate Buffer)

Carbon Source	5.00 g
$(NH_4)_2SO_4$	1.18 g
NaCl	0.50 g
$MgSO_4 \cdot 7\ H_2O$	0.50 g
$CaCl_2 \cdot 2\ H_2O$	0.20 g
K_2HPO_4	2.00 g
KH_2PO_4	1.00 g
Trace Metals, ea. (Fe, Mn, Zn, Co, Mo, Cu)	0.50 mg
Demineralized Water	1.00 liter

The solution was autoclaved, cooled and sterile solutions of iron, zinc and phosphate were added. The phosphate solution was autoclaved, but the iron and zinc solutions were sterilized by filtration. This was necessary to prevent oxidation of the ferrous iron and hydrolysis of both the iron and zinc. In addition, the iron was maintained in the reduced state by acidifying the solution and storing in the dark. The prevention of this chemical precipitation was extremely important because growth was completely inhibited when precipitation occurred.

As the initial work progressed, the fungi would produce growth in the media being used. However, this growth was poor and very slow. This may have been expected of fungi, particularly in the sodium acetate medium. The growth in glucose was better and not abnormal. However, during this period of study, an observation was made which suggested a change in the buffer system would be desirable. Several flasks of media had been prepared, and all were not innoculated. The flasks without innoculum developed a floc or precipitate. There should not have been any contamination and a microscopic examination was made to determine the characteristics of this floc. The particles were not microorganisms, but chemical crystals. It appeared that even though the initial precipitation was eliminated with the media preparation technique used, precipitation did occur in time. The formation of precipitate was masked by the turbidity created by the microorganisms in the innoculated cultures.

As a result of this observation and the apparent slow growth of fungi on this media, the bicarbonate buffer system was substituted for the phosphate buffer system. The media composition is given in Table 6.

TABLE 6

Substrate Composition - Pure Culture Studies (Bicarbonate Buffer)

Carbon Source	5.00 g
$(NH_4)_2\ SO_4$	1.18 g
NaCl	0.50 g
$MgSO_4 \cdot 7\ H_2O$	0.50 g
$CaCl_2 \cdot 2\ H_2O$	0.08-0.20 g
KH_2PO_4	0.20 g
$NaHCO_3$	3.00 g
HCl (Adjust pH to 7.0)	
Trace Metals, ea. (Fe, Mn, Zn, Co, Mo, Cu),	0.5 mg
Demineralized Water	1.00 liter

The media was prepared by autoclaving all constituents excepting the bicarbonate, iron and zinc. The iron and zinc were sterilized and stored as before. The bicarbonate solution was sterilized by filtration and added to the sterile media. Hydrochloric acid was added to the substrate in such quantity to produce a pH of 7.0 after addition of the bicarbonate buffer. This quantity of acid varied with the carbon source, and was determined by experimentation.

3. Seeding of Test Flasks - Phase 1

After the media had been prepared and the proper metals added, the flasks were seeded with the test organism. The seed culture was prepared by innoculating the media to be tested with the organism maintained on the mycological agar. The seed culture media contained the carbon source plus all of the metal cations to be tested. Sufficient time was allowed for an abundant growth to develop in the seed culture. The culture was examined microscopically for contamination. The test flasks were then seeded with the pure culture.

The volume of the media in each flask was 300 ml. To this was added 5 ml of seed. This provided an ample innoculum and a minimum carry-over of trace metals. The original metal concentrations in the seed cultures were 0.5 mg/l. By diluting this concentration by a factor 60, the metal concentration in the test cultures resulting from carry-over from the seed culture was about 8 μg/l.

Realizing that the above concentration may be adequate to meet the nutrient requirements of the fungi, a second set of test media were prepared. This time, the seed was taken from the previous test run, using the appropriate flask to seed the flask in which the individual metal was eliminated. This provided another dilution factor of 60 which reduced the concentrations of the metals from the original seed culture to 0.1 μg/l. This concentration should be lower than the minimum nutrient requirement and also is in the range of solubilities of metal ions in almost any chemical system. It is highly improbable that these common metal cations would not be present at this concentration range, no matter what the source of waste water.

4. Growth Conditions - Phase 1

The test flasks were placed on a New Brunswick Model G-10 gyrotory shaker and shaken at 200 cycles per minute. This provided very good agitation, and maintained the cultures aerobic. Sterile conditions were maintained during the test period with cotton plugs in the flask. Care was exercised in all transfers and sampling of culture to prevent contamination. All test flasks were examined microscopically at the end of each run and the data from any contaminated run were discarded.

Temperature control was not strict because only the relative growth was considered. The room temperature was about 20°C. The room was heated and air conditioned which maintained a reasonably constant temperature.

All glassware used in these experiments were Pyrex brand glass. The glassware was thoroughly washed in chromic-acid cleaning solution with multiple rinses using tap and then demineralized water. This was done to minimize adsorption of metals onto the glass with subsequent release to the microorganisms.

5. Measurement of Growth Response - Phase 1

The growth response of the fungi was ascertained by measuring the changes in cell mass with time. This was done by filtering a 10 ml sample of the culture through a membrane filter having a pore size of 0.45 μ. This technique is the one proposed by Engelbrecht and McKinney (36) and was selected because of the ease in evaluating the growth of the microorganisms. Such techniques as optical density could not be applied to these cultures because of the non-homogeneity of the culture. The fungi form very discrete floc particles causing agglomeration of the dispersed particles. This prevents measurement of the growth of the fungi as indicated by an increase in optical density.

When measuring the sludge growth, the flasks were removed from the shaker. Sample volumes of 10 ml were taken from the flasks after careful mixing. Because of the filamentous nature of the growths, a wide mouth volumetric pipet was used. Even so, obtaining a representative sample was difficult. This sampling problem was responsible for sample variations of as much as 20 to 30%. Therefore, it is only when a series of samples show the same effect, that any confidence can be placed in the data. To overcome this problem several measurements were made with time, establishing the growth curve.

6. Experimental Units - Phase 2

This phase of the project was conducted in laboratory activated sludge units. These systems consisted of a 7 liter aeration compartment followed by a 0.5 liter settling compartment. The soluble substrate was fed by a constant volume feed pump at the rate of 145 ml/hr providing an aeration time of 48 hours. The settling time was 3.5 hours, having a surface loading on the settling tank of less than 100 gpd/ft^2. This was thought to be reasonable on this scale, with very efficient solids removal resulting when dealing with a good settling sludge. Even poor settling sludge was effectively removed in this system. Figure 6 is a schematic of the laboratory units used.

Aeration was accomplished with compressed air. This air was first passed through a water trap to collect any condensate from the air lines. The air was also bubbled through water to minimize the evaporation of the water from the units. Small porous diffuser stones were used to obtain efficient oxygen transfer. The rate of aeration was always in excess such that the dissolved oxygen in the system was at least 2 mg/l. This was periodically checked with a dissolved oxygen probe. The temperature remained reasonable constant at 20°C. The only temperature control was that available through heating and air conditioning of the laboratory.

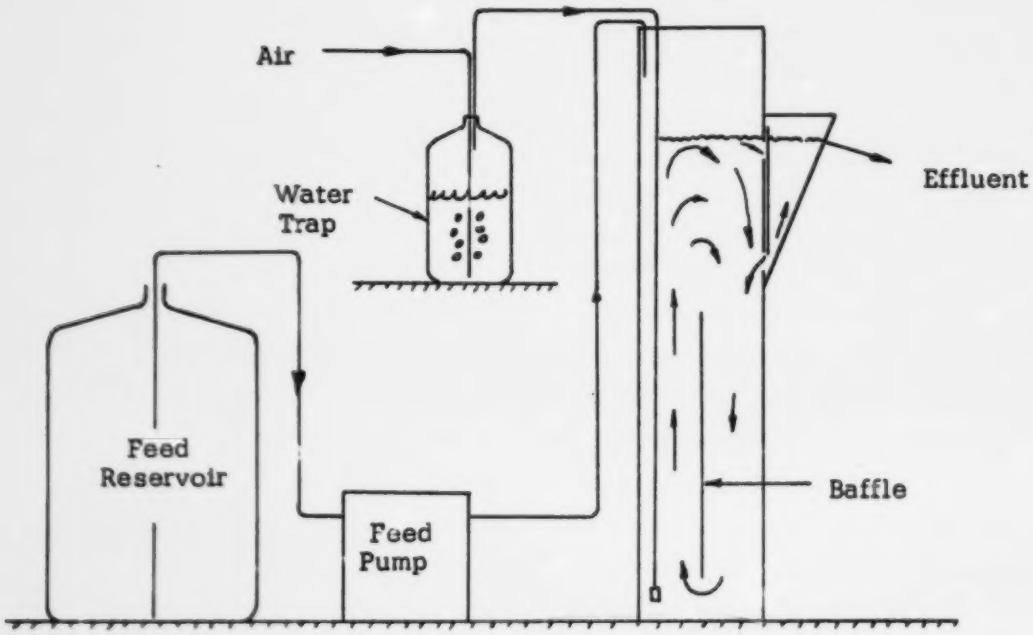
The systems were started from raw domestic sewage from the Lawrence treatment plant. The sewage was aerated for 48 hours. The settleable solids were allowed to settle and tow-thirds of the supernatant was withdrawn. This volume was replaced with the substrate of the composition shown in Table 7. This procedure was repeated until the sludge flocculated sufficiently to be removed in the settling tank. Continuous feeding was then initiated and the units subjected to the test conditions. Until this time, the system received a balanced substrate containing all of the trace metals. When a new substrate was introduced, this procedure was repeated using raw sewage as the initial seed of organisms.

7. Substrate - Phase 2

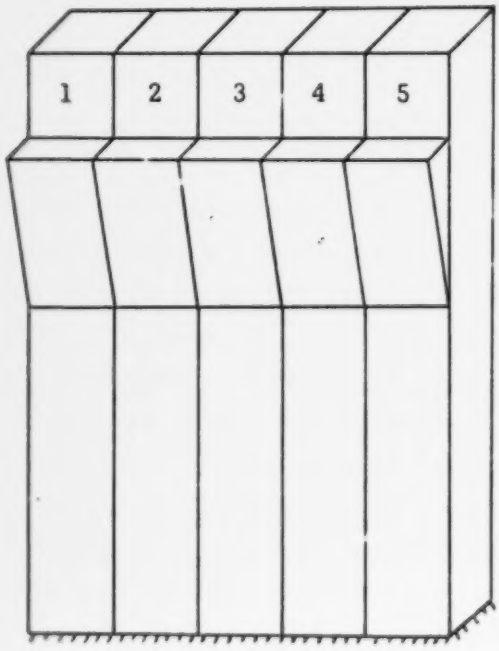
The substrate used in this phase is given in Table 7. Both sodium acetate and glucose were used as the carbon source. The trace metals were added individually and in various combinations and were the test conditions to which the systems were subjected. The chemicals used were all analytical grade to minimize the contamination with trace metals.

FIGURE 6

SCHEMATIC DIAGRAM OF LABORATORY UNITS



Side View



Isometric View

TABLE 7

Substrate Composition - Phase 2

Carbon Source	2.00 g/l
$(NH_4)_2\ SO_4$	0.47 g/l
$KH_2\ PO_4$	0.02 g/l
$CaCl_2\ ;\ 2H_2O$	0.73 g/l
Trace Metals, ea.	0.5 mg/l
Demineralized Water	

This substrate produced a loading on the system of 62.5 lb BOD/day/1000 cu. ft. At mixed liquor solids concentration of 3000 mg/l, the BOD to solids loading is 0.33. This is very typical of a conventional activated sludge system. Higher mixed liquor solids will reduce this loading more toward the range of an extended aeration system. The volumetric loading is greater than normal but the solids were carried at a higher level than normally carried in an activated sludge system.

At this loading, the sludge production would be about 0.5 lbs of sludge per lb of 5-day BOD. This would produce about 2.5 g of sludge solids per day resulting in an increase in the mixed liquor solids of 300 to 400 mg/l per day. This will occur only when metabolism of the substrate is complete. With a sludge production of 2.5 g per day, about 0.3 g of nitrogen will be required. The substrate contained 100 mg/l of nitrogen and at a feed rate of 3.5 l/day, the available nitrogen would be 0.35 g which is more than adequate.

The phosphorus required for this sludge production would be between 0.025 and 0.05 g. With a phosphorus concentration of 5 mg/l in the feed, the available phosphorus would be 0.018 g. This would appear to be insufficient. However, analysis of the effluent showed soluble phosphorus values of a few tenths of a mg/l. This element was intentionally maintained at a low level to minimize the interaction between the metal cations and phosphate. Frequent phosphorus analyses of the effluent were conducted to be sure that phosphorus was not limiting the microorganism growth.

The control of pH was a very significant factor in these experiments. In the tests using acetate, a mixture of acetic acid and sodium acetate was used as the carbon source. The ratio of this mixture was varied to maintain the pH at about 7.0. Because of the differences in substrate metabolism

with the different metal combinations, it was not always possible to control the pH with the feed. The pH of the untis were checked daily and either sodium bicarbonate or sulfuric acid was added for pH adjustment. The pH of the units did not fluctuate more rapidly because of the 48 hour retention time employed.

With glucose, the pH was partially controlled by addition of sodium bicarbonate to the substrate. Care had to be exercised because the addition of too much bicarbonate to the feed would elevate the pH in the feed above 8.0. Small amounts of precipitate could be observed. Therefore, only limited amounts of bicarbonate were added to the substrate, the rest was added to the units as needed to maintain a pH of about 7.0.

8. Measurement of Growth Characteristics - Phase 2

The growth response of the mixed cultures was measured by the substrate removal and settleability of the resulting sludge. Under the conditions employed in these tests, effective substrate removal was considered indicative of good growth. The substrate removal was evaluated on the basis of soluble COD removal. The substrate removal did not indicate the type of organisms produced. The characteristic most significant to this work is the sludge volume index. This is an indication of bulking sludge. Therefore, this parameter was used to evaluate the settleability of the sludge. All tests except suspended solids were conducted according to Standard Methods (39). The suspended solids were conducted according to the method of Engelbrecht and McKinney (38).

Samples for these analysis were collected at the time of analysis. The effluent samples were collected for about a 3 hour period producing a sample volume of about 450 ml. The samples were then filtered and analysed. Mixed liquor samples were taken from the aeration tanks after cleaning the tank walls and thoroughly mixing the tank contents. These samples were analysed for suspended solids and settleable solids.

Before data was collected, the units were allowed to come to equilibrium. A two week period was allowed for this purpose. When changing the test conditions, the units were reseeded with raw sewage to assure the presence of a variety of microorganisms. This was necessary because certain of the metal combinations essentially inhibited all growth while other combinations produced basically a pure culture of organisms.

EXPERIMENTAL RESULTS - PHASE 1

The initial experiments proved to be very frustrating because of the rate of growth of the microorganisms on the media given in Table 5. It was possible to prepare the media such that no metal precipitation occurred initially. However, the precipitates did occur after a period of time. Figure 7 shows the growth rate of the fungi, Fusarium with acetate as the carbon source. The growth response to the absence of various metals was very poor as shown by the growth rate in the control flask. The flask not containing copper appeared to have a higher growth rate. The inhibition in growth of this fungus was at first attributed to the presence of the trace amounts of copper.

1. Chemical Interactions with Phosphate

To check the effect of copper, a run was made eliminating the copper from all samples. These data are shown in Figure 8. The growth response improved somewhat. However, the growth rate was still very slow. The effects of eliminating iron and cobalt in addition to copper can be seen from the appropriate curves. At about this time, media that had been standing for several days without innoculation began to show precipitation of something. It was suspected that the high concentrations of phosphates used in the buffer system were reacting with the metal ions. To check this possibility, the bicarbonate buffer system was substituted for the phosphate system. The composition of this media is shown in Table 6.

The increase in the growth rate was tremendous. Figure 9 is a plot of the increase in mass of Fusarium in a actetate medium employing bicarbonate as the buffer. Also, copper was eliminated from this medium. In the first two runs, maximum growth was not achieved in 10 days. However, when the high concentration of phosphates was eliminated, yields of over 2.0 g/l of fungal mass was obtained in less than 48 hours. It became apparent that the media would have to be changed. The remaining pure culture studies with Fusarium were conducted with the media given in Table 6, with the variations in the calcium concentration. Media deficient in cobalt, manganese, or molybdenum produced the same growth response as the control medium which contained all 5 metals. Iron or zinc deficiencies did retard the growth as shown in Figure 9.

FIGURE 7

FUSARIUM GROWTH ON ACETATE MEDIA
PO_4 BUFFER, CU IN MEDIA

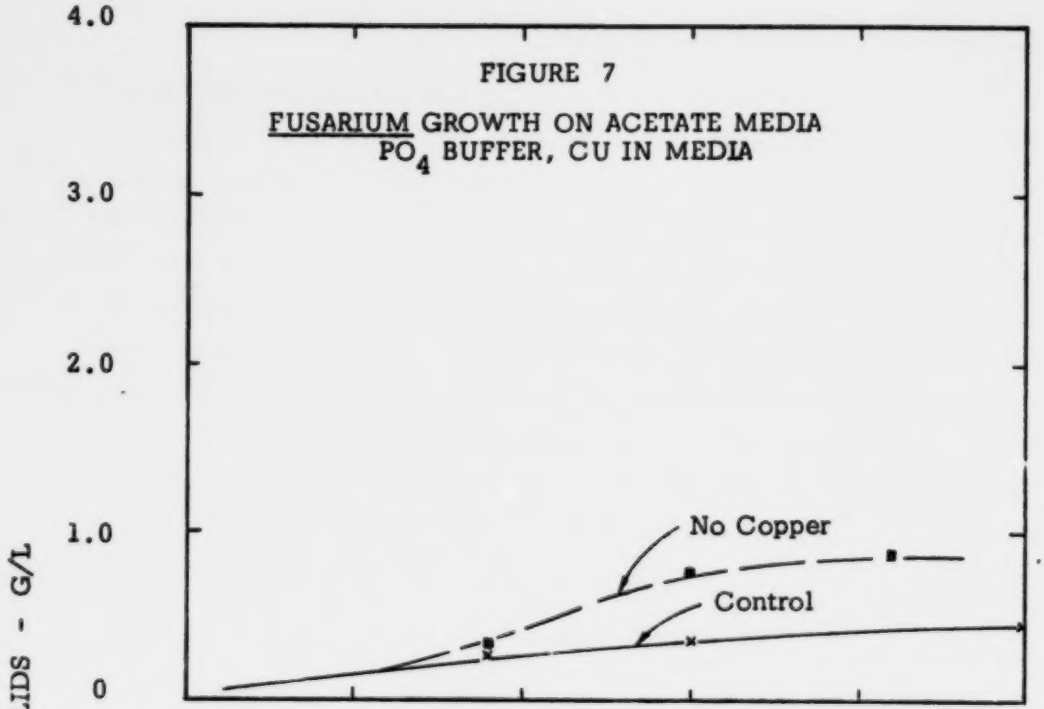
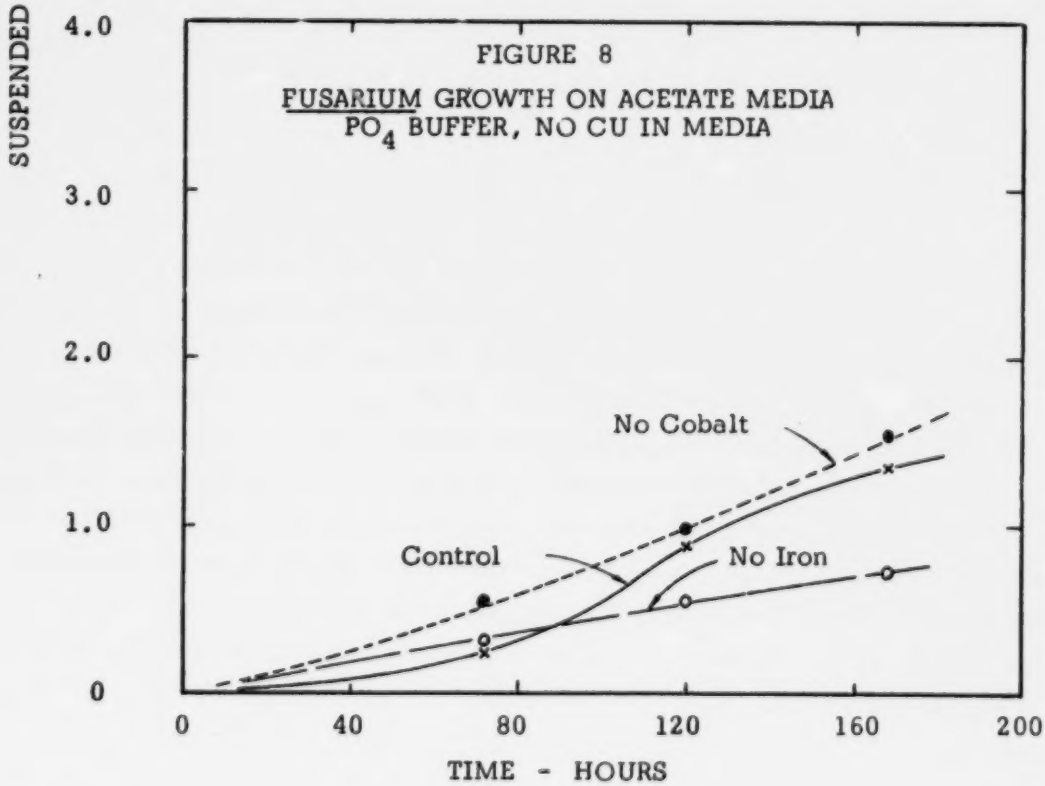


FIGURE 8

FUSARIUM GROWTH ON ACETATE MEDIA
PO_4 BUFFER, NO CU IN MEDIA



2. Calcium and Magnesium Requirements

To check the requirements of Fusarium for calcium and magnesium, several runs were made using sodium acetate and eliminating copper from the medium. These results are shown in Figure 10. Calcium did not appear to be significant in the growth of the organisms in the medium which contained magnesium. There was growth inhibition at calcium concentrations in excess of 20 mg/l. This was probably a result of the precipitation of the phosphate ion which is present in the medium at low concentrations. With sodium acetate as a substrate, the pH would increase to about 9.0 even with the buffer system being used. At this pH , a considerable amount of interaction between calcium and phosphate can be expected. This test was repeated producing similar results. These data are given in Table 8.

TABLE 8

EFFECT OF CALCIUM CONCENTRATION ON THE GROWTH OF FUSARIUM ON SODIUM ACETATE MEDIUM

Time	Calcium Concentration - mg/l					
hrs	0	10	20	30	40	50
0	0	0	0	0	0	0
20	300	380	380	300	430	300
42	940	1090	1100	990	-	390
90	1780	1580	1840	1860	1580	760

With another carbon source that does not increase the pH when metabolized as does sodium acetate, the effect of calcium concentrations in excess of 20 mg/l may not be adverse. To check this, glucose was substituted for the acetate and all trace metals except copper were present. The results are given in Table 9. Again, the presence or absence of calcium does not appear to have any effect on the growth response at 0 to 30 mg/l of calcium. At the high clacium concentrations, the growth response appears to be greater, indicating the desirability of higher calcium concentrations when the pH is not elevated sufficiently high to cause calcium phosphate precipitation.

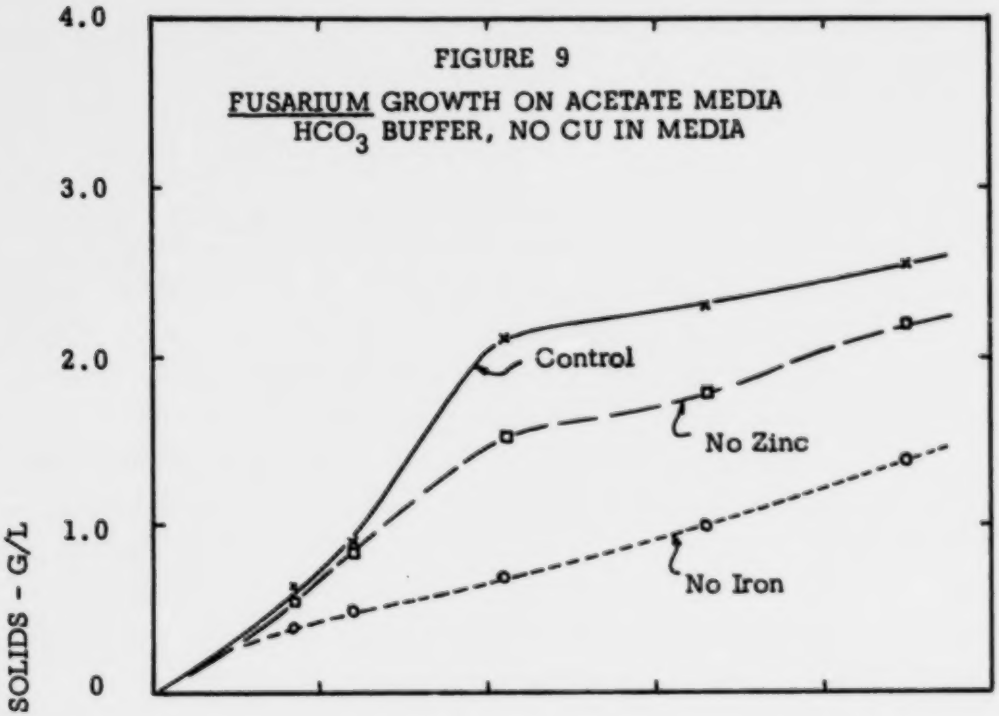


FIGURE 9

FUSARIUM GROWTH ON ACETATE MEDIA
HCO_3 BUFFER, NO CU IN MEDIA

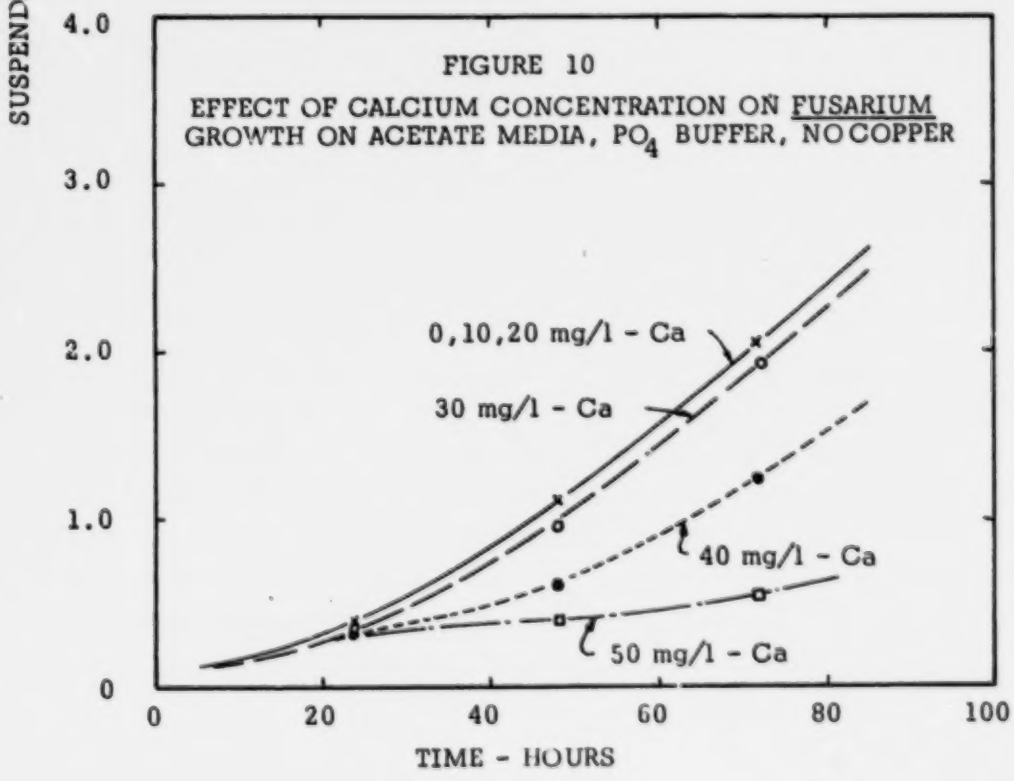


FIGURE 10

EFFECT OF CALCIUM CONCENTRATION ON FUSARIUM
GROWTH ON ACETATE MEDIA, PO_4 BUFFER, NO COPPER

TABLE 9

EFFECT OF CALCIUM CONCENTRATION ON THE GROWTH OF FUSARIUM ON GLUCOSE MEDIUM; 4-DAY CULTURE

Ca mg/l	0	20	30	40	50
Growth mg/l	2040	2180	1970	2600	3110

In most cases, waters will contain 40 mg/l or less of calcium. Since there was little effect on the growth response at concentrations below 40 mg/l, a value of 20 mg/l of calcium was selected for use in these media. This will eliminate any problem of phosphate precipitation at an elevated pH.

To check on the optimum magnesium concentration at a calcium level of 20 mg/l, data were collected at various magnesium levels using Fusarium and the glucose medium. These data are given in Table 10. Zero mg/l of magnesium appeared to reduce the growth response. Levels of 10 to 120 mg/l did not produce any major increase in the growth responses. It would appear that at least 10 mg/l of magnesium is required for growth of this fungi.

TABLE 10

EFFECT OF MAGNESIUM CONCENTRATION ON FUSARIUM GROWTH ON GLUCOSE MEDIUM

Time	Magnesium Concentration - mg/l					
hr	0	10	20	40	80	120
0	0	0	0	0	0	0
24	270	170	250	-	410	450
48	1020	1730	1410	1510	1720	1440

To provide for additional evaluation of the effects of the combination of the calcium and magnesium of the growth response, data were collected on the growth response of Fusarium to different combinations of calcium and magnesium. The carbon source was glycerol. These data are shown in Table 11. The only significant effect shown by these data is the poor growth when the magnesium was zero. All other combinations appeared to produce approximately the same growth response.

TABLE 11

RESPONSE OF FUSARIUM TO CALCIUM AND MAGNESIUM WITH GLYCEROL AS THE CARBON SOURCE

Ca mg/l	Mg mg/l	Growth (mg/l)
50	50	3140
0	50	2840
0	25	3500
25	25	3600
50	0	1350
25	0	890

Since there was no apparent reaction between the high magnesium concentrations and the various anions, the magnesium concentration was left at 50 mg/l. However, the calcium content was lowered to 20 mg/l to prevent the calcium phosphate interaction at the high pH levels obtained with sodium acetate as the carbon source.

3. Growth of Fusarium on Acetate Media

Using sodium acetate as the carbon source for the medium given in Table 6, the growth of Fusarium as related to the availability of trace metals was determined. The data in Figure 11 were obtained from flasks seeded from a common seed flask containing all trace metals except copper. The lack of iron has a tremendous effect on the growth response of this organism. Absence of zinc reduced the growth of the fungus, but not as severly as the absence of iron.

To check additionally on the trace metal needs of this organism, a second run was made in which the seed was obtained from the previous run. The seed for the flask without iron was taken from the previous flask which did not have any iron. This procedure was followed for all flasks. The purpose was to minimize the carry-over of trace metals from the seed cultures. The original seed contained 0.5 mg/l of the metals. Transfer of 5 ml of this seed to 300 ml of medium without a given metal reduced the concentration to about 8μ g/l. Seed from the flasks containing the 8μ g/l was transferred to the second 300 ml of medium, reducing the concentration to about 0.1μg/l. This concentration was assumed to be below the level required as a nutrient.

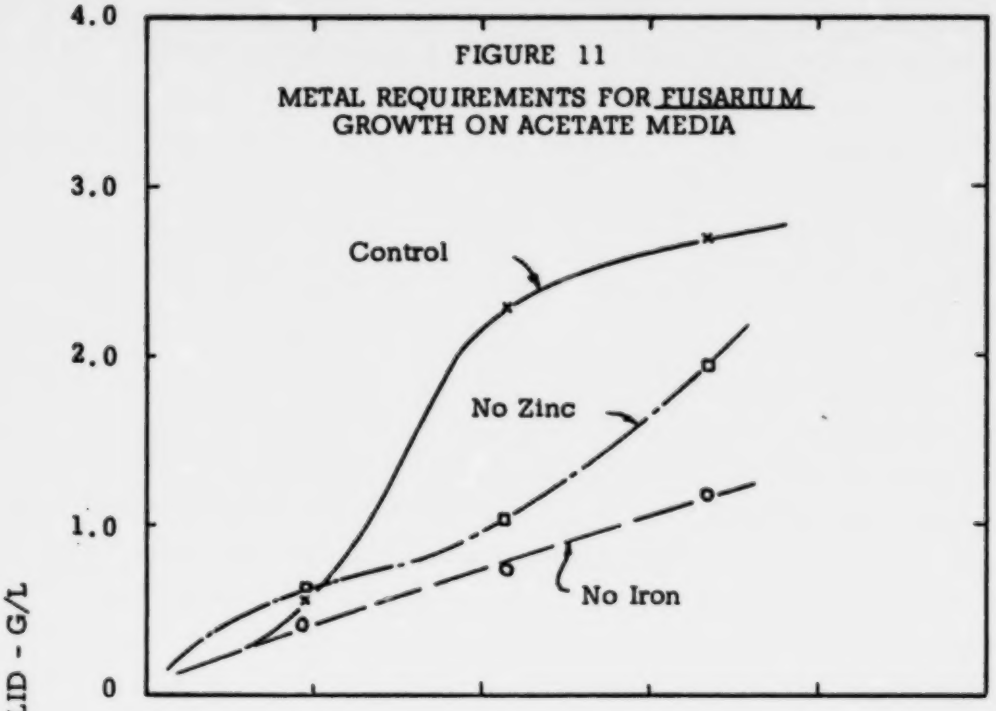


FIGURE 11

METAL REQUIREMENTS FOR FUSARIUM GROWTH ON ACETATE MEDIA

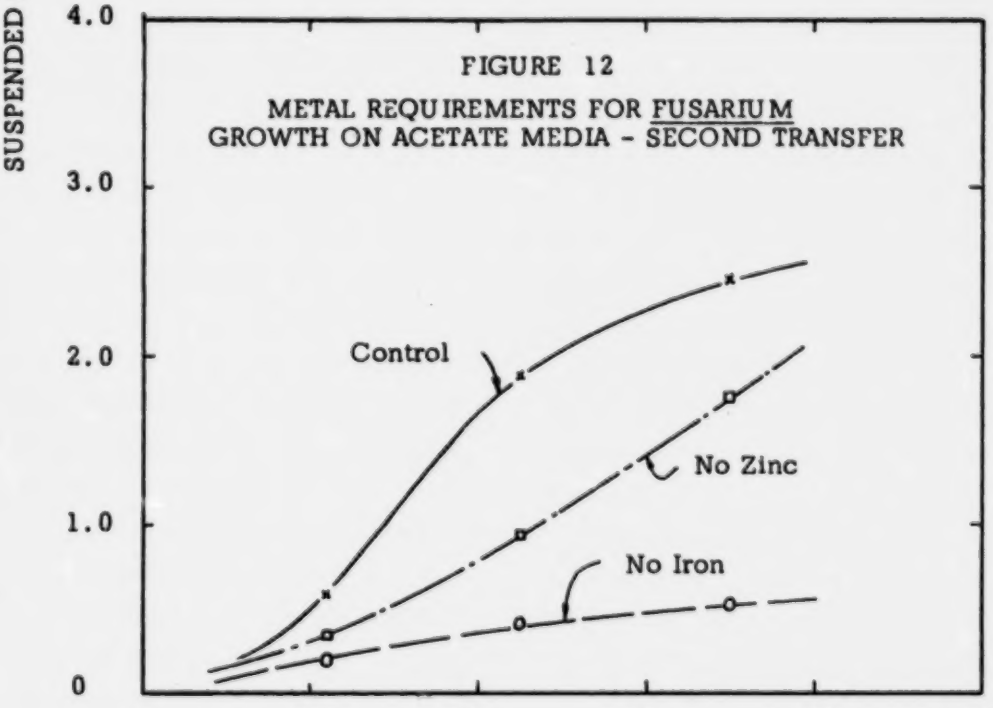


FIGURE 12

METAL REQUIREMENTS FOR FUSARIUM GROWTH ON ACETATE MEDIA - SECOND TRANSFER

SUSPENDED SOLID - G/L

0 20 40 60 80 100

TIME - HOURS

The results of this second transfer are shown in Figure 12. The effect of no iron in the medium was very significant, even more so than in the first transfer. The response to the absence of the remaining metals was not changed indicating that only iron is required for the growth of Fusarium on this medium. The growth response to the medium without zinc was retarded, but not as severely as the iron deficient medium.

4. Growth of Fusarium on Glucose Media

Glucose was used as the carbon source in these runs. Figure 13 shows the growth response of Fusarium to this medium as listed in Table 6. The growth was very rapid in all cultures excepting those in which iron and zinc were missing. There was a definite inhibition in Fusarium growth when either of these two metals were missing. The maximum growth was as high as the control, but the time required to obtain this growth was significantly longer.

The effect of copper on the growth of Fusarium on the glucose was checked by a run in which copper was used in the medium. The growth response is shown in Figure 14. The response was rather unusual. Growth in the control flask was marginal with good growth in the flask containing no copper. To further check this effect, a second run was made using this run as seed. The seed for the flask without iron was taken from the previous culture containing no iron and so forth for the remaining flasks. The growth response to these media is shown in Figure 15.

The results of this test is very interesting. The flask without iron produced reasonable good growth, as did the flask without copper. All flasks containing copper and iron were definitely inhibited as shown by the control. This can only be explained as an antagonistic effect of these two metals. It would appear that the copper inhibits the growth of Fusarium when in combination with iron.

5. Growth of Fusarium on Glycerol Media

In these runs, glycerol was the carbon source in the medium listed in Table 6. Copper was used in the medium to evaluate its effect on Fusarium growth on glycerol. These data are shown in Figure 16. The

FIGURE 13

N :TAL REQUIREMENTS FOR FUSARIUM GROWTH ON GLUCOSE

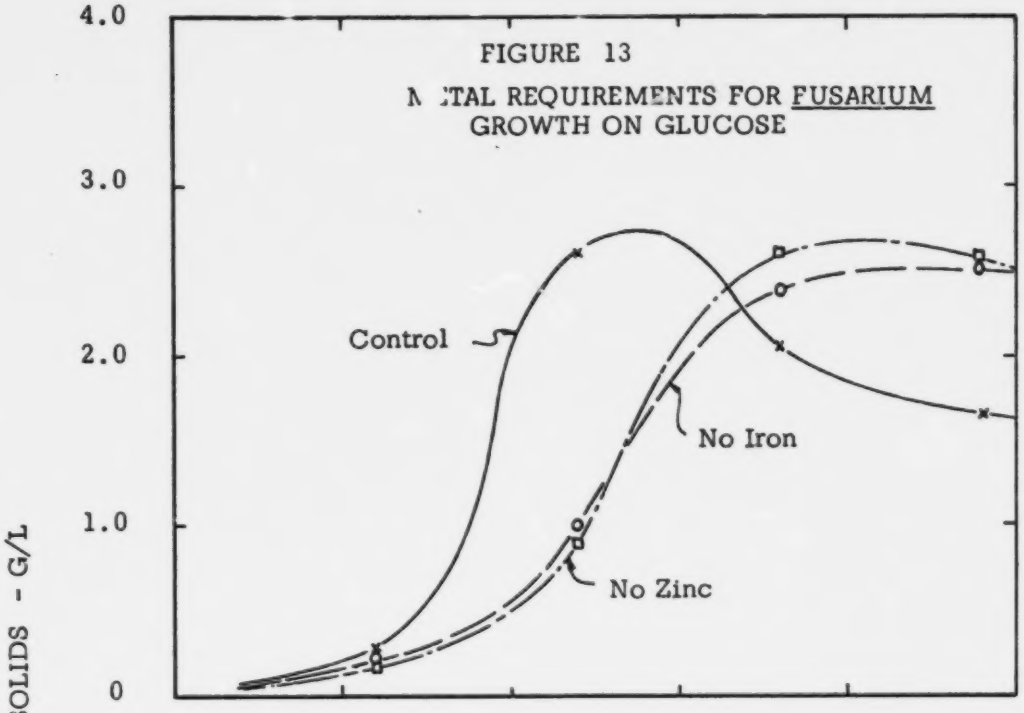
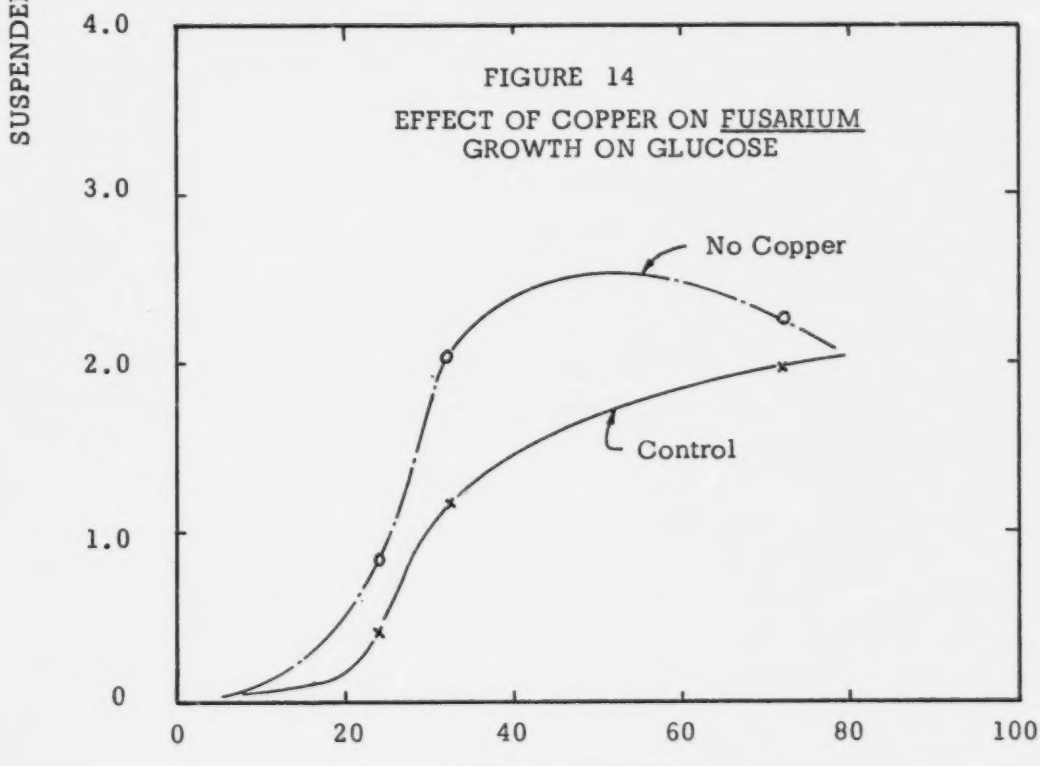


FIGURE 14

EFFECT OF COPPER ON FUSARIUM GROWTH ON GLUCOSE



SUSPENDED SOLIDS - G/L

control growth response was very poor. However, flasks containing no iron, no copper or no molybdenum yielded good growth. To determine the effect of metal carry-over, another run was made using this initial run as seed for the flasks which did not contain the various metals. These effects are shown in Figure 17.

Again the metal carry-over is evident. In the second transfer, all flasks containing both iron and copper produced very poor yields. The flask without copper produced excellent growth. The flask without iron was substantially better than the control as well as better than the rest of the flasks. The inhibition of copper is evident, but when iron is missing, the copper inhibition is not as significant. This would also indicate an antagonistic effect of copper and iron in glycerol media.

Since copper appears to have an inhibiting effect on Fusarium growth on glycerol, it was eliminated from the media. Figure 18 is a plot of the growth response of Fusarium on glycerol medium that does not contain copper. The growth was very good in all flasks, as indicated by the control, except the one without iron. This suggests that only iron is required for the growth of Fusarium in this medium.

6. Suspended Solids Composition and pH Changes

Because of the large variations in pH with the different media, the possibility of the suspended solids measuring more than cell growth was investigated. With sodium acetate as the carbon source, the final pH levels encountered in these tests were in the range of 9.0. Conversely with the glucose and glycerol, the final pH values were in the order of 2.0 to 3.0. This low pH would generally improve solubility of most compounds while the high pH could cause precipitation of many materials. The suspended solids were 90 percent or more volatile for the glucose and glycerol substrates. However, on the sodium acetate medium 25 to 30 percent of the solids production may be a result of chemical precipitation, particularly at the end of the runs when the pH would increase to levels of 9.0 (see Table 12).

The very low pH levels found with the glucose and glycerol media would appear to be caused by mineral acid production rather than organic acid. To check this, an analysis was made of the soluble COD in the flasks.

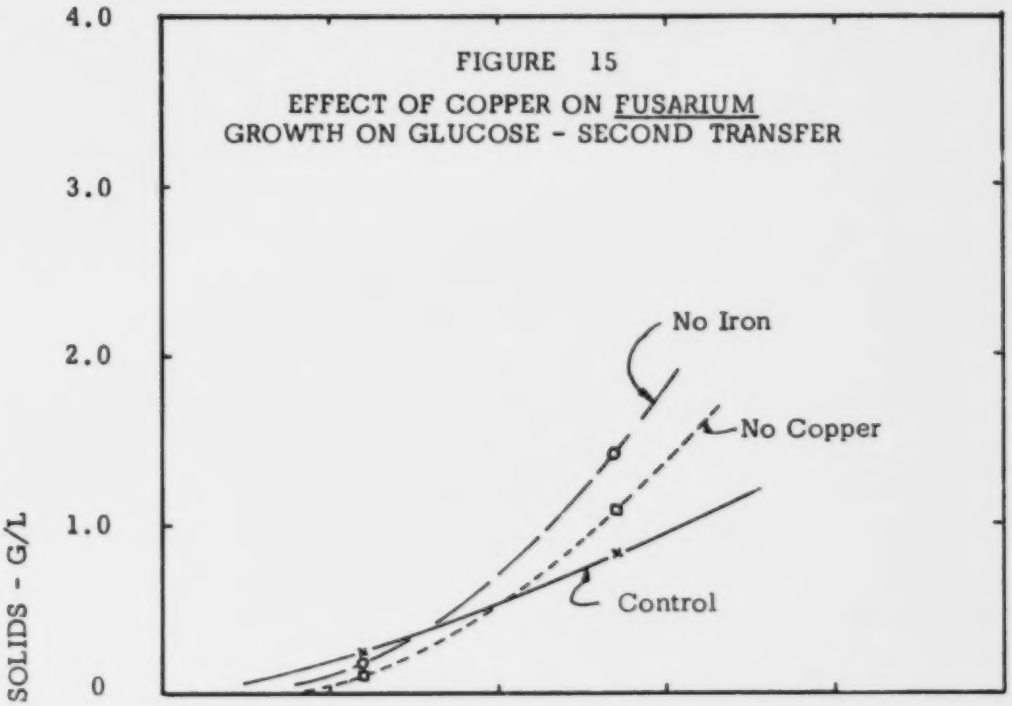


FIGURE 15

EFFECT OF COPPER ON FUSARIUM
GROWTH ON GLUCOSE - SECOND TRANSFER

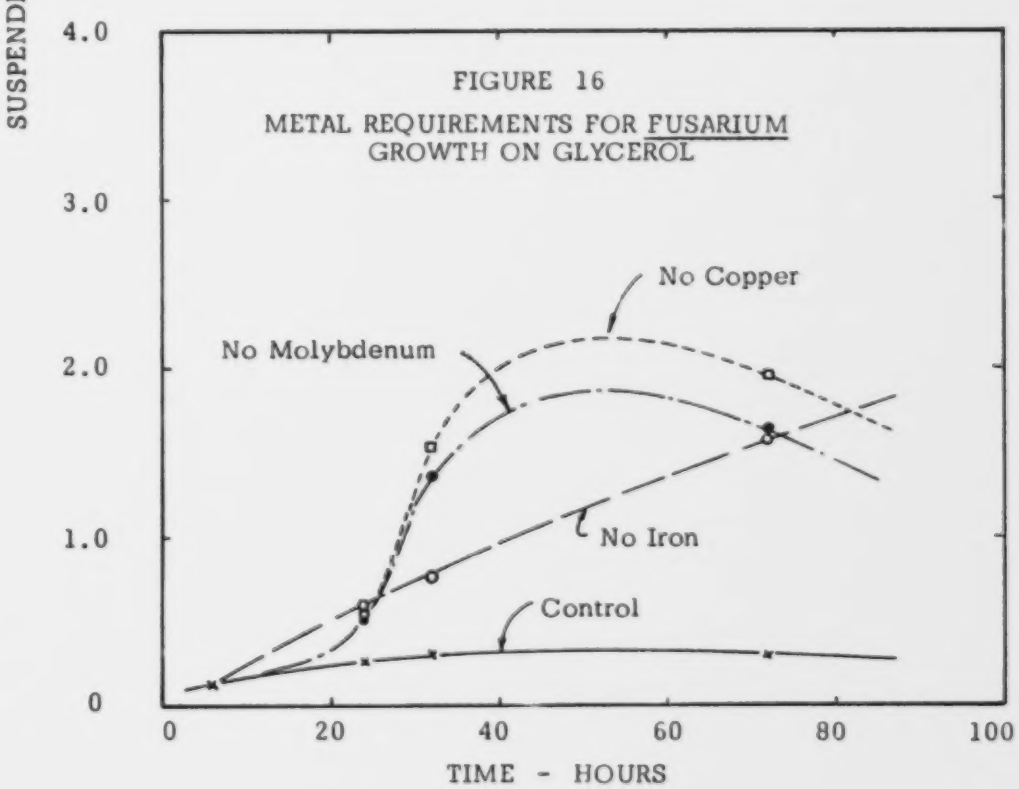


FIGURE 16

METAL REQUIREMENTS FOR FUSARIUM
GROWTH ON GLYCEROL

FIGURE 17

METAL REQUIREMENTS FOR FUSARIUM GROWTH ON GLYCEROL - SECOND TRANSFER

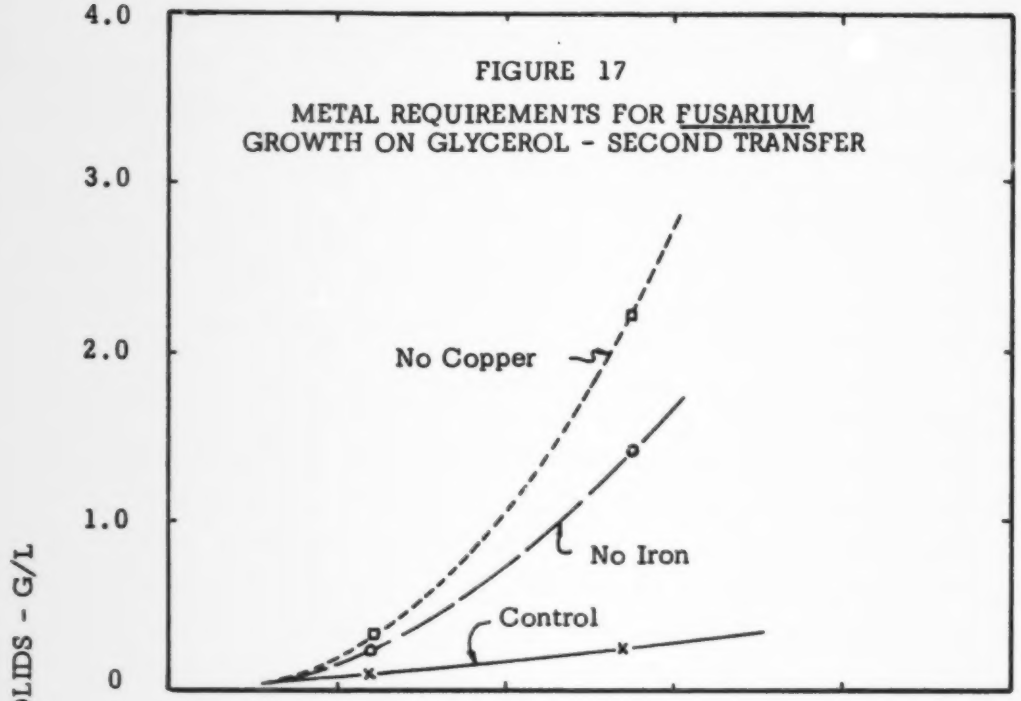


FIGURE 18

FUSARIUM GROWTH ON GLYCEROL MEDIA WITHOUT COPPER

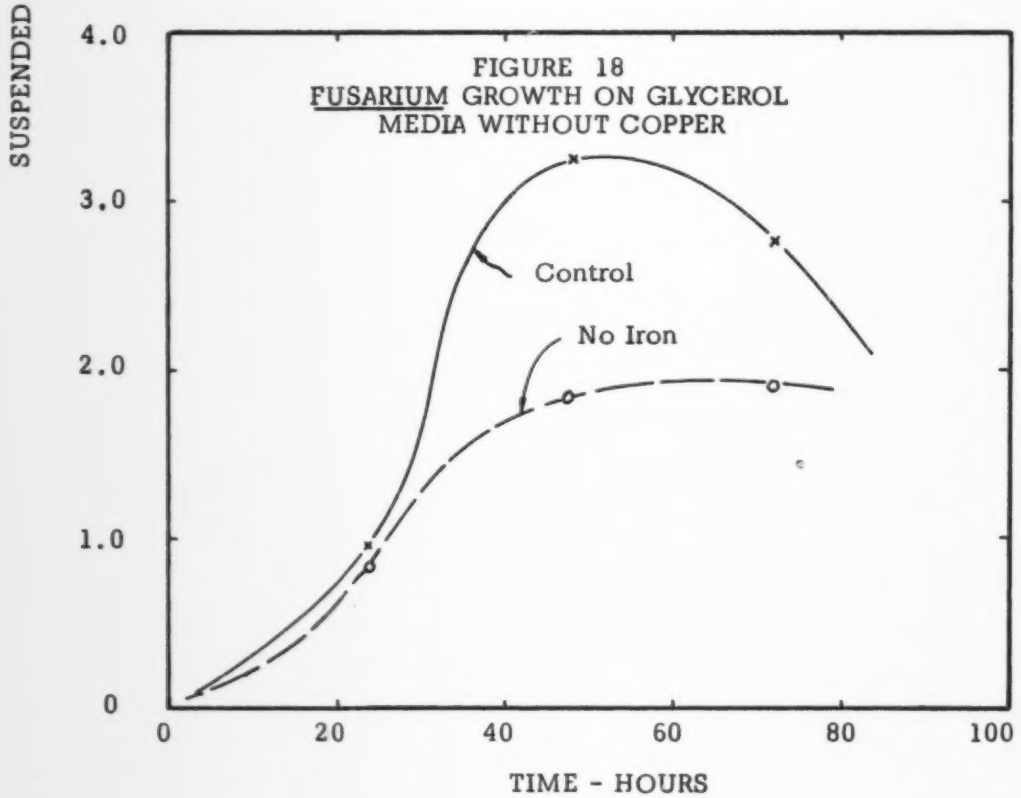


TABLE 12

VOLATILE SOLIDS COMPOSITION OF FUSARIUM CULTURES

Media	Final pH	Tot. S.S.	Fixed S.S.	Vol. S.S.	%Vol. S.S.
Sodium Acetate	9.5	1990	600	1390	70.0
Glucose	2.0	3390	200	3190	94.0
"	2.5	2330	150	2180	93.5
Glycerol	2.0	4880	370	4570	92.5
"	3.0	1950	180	1770	90.6

If organic acids were involved, they could exert a COD. Table 13 shows the COD relationship in the flasks. Better than 90 percent of substrate COD had been removed. This is also indicated by the growth of fungi. The suspended solids production was generally sufficient to account for metabolism of all of the substrate. This was particularly true when the pH was very low. Therefore, the pH drop must result from the production of hydrogen ions from the metabolism of the substrates.

TABLE 13

SOLUBLE COD REMAINING AFTER GROWTH OF FUNGI

Substrate	Initial COD, mg/l	Soluble COD, mg/l
Glucose	5350	436
Glucose	5350	121
Glucose	5350	255
Glycerol	6100	300
Glycerol	6100	230
Glycerol	6100	230

7. Growth of Geotrichum on Acetate Media

In addition to Fusarium, Geotrichum was isolated from the waste water. This organism has been associated with sludge bulking and as such, its growth requirements are of considerable interest. The growth response

of Geotrichum on sodium acetate medium was ascertained. These data are shown in Figure 19. The growth rate is low, but all flasks produced reasonable growth except the one without iron and the one without zinc. In addition, the flask without copper gave a substantially better growth response than any flask containing copper. This again suggests an inhibition of this fungal growth by copper.

A check was made on the effect of metal carry-over from the seed culture by seeding another set of flasks with seed from the appropriate flask from the previous run. That is, the seed for the flask without iron was taken from the flask that did not contain iron in the last run, etc. This effectively dilutes any carry-over of the metals from the original seed culture. These data are shown in Figure 20. The growth was markedly reduced in all flasks. Unfortunately, the flask without copper was contaminated and lost. Also, the flask without cobalt was contaminated. It appeared that copper and possibly cobalt were inhibiting growth, particularly with the complete washout of the trace metals or some other factor from the seed culture.

The need for both cobalt and copper was checked by a run in which both copper and cobalt were eliminated from the medium. These data are given in Figure 21. The growth response was almost as good as the flask without copper in Figure 19. The absence of both cobalt and copper did not appear to limit growth. In fact the growth was as good if not better than when these metals were present.

8. Growth of Geotrichum on Glucose Media

Glucose was substituted for acetate in the basic media and inoculated with Geotrichum. The growth response for the first inoculation is shown in Figure 22. The growth was very good in all cases. There was no significant inhibition by the presence or absence of any metal. On the chance that the carry-over from the seed culture provided sufficient trace metal, a second dilution was made by transferring seed from the individual flasks in this run to the appropriate flasks in the next run. These data are shown in Figure 23.

The metal carry-over from the seed culture was indeed adequate in the first dilution to satisfy the nutrient requirements of the fungus. The second dilution showed that only zinc was important in the nutrition of

FIGURE 19

METAL REQUIREMENTS FOR GEOTRICHUM GROWTH ON ACETATE MEDIA

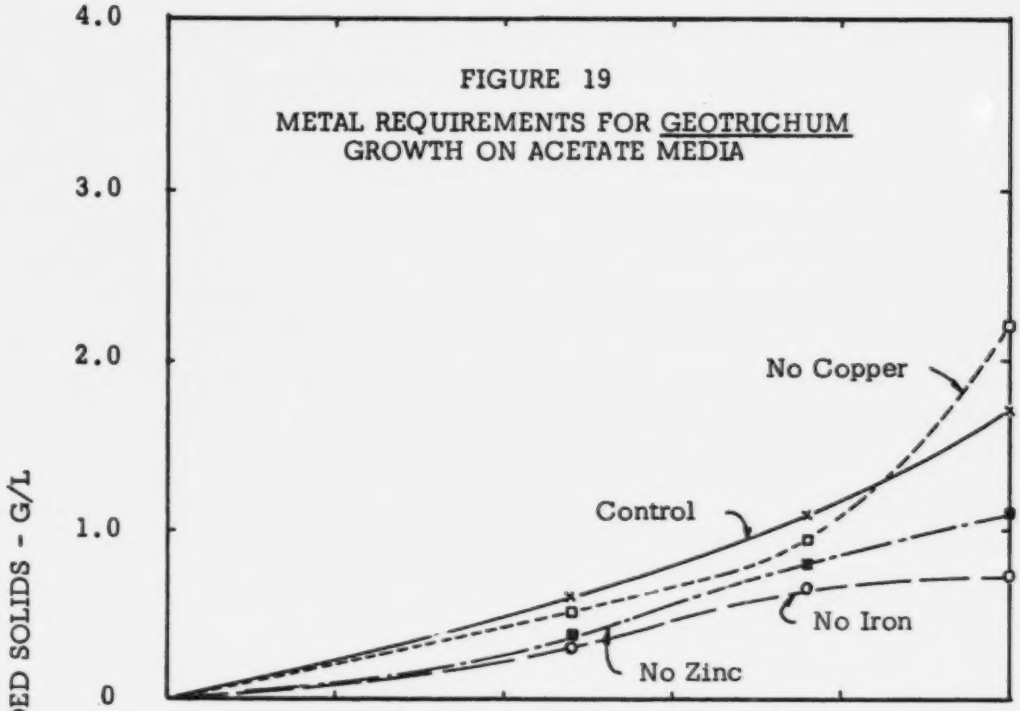
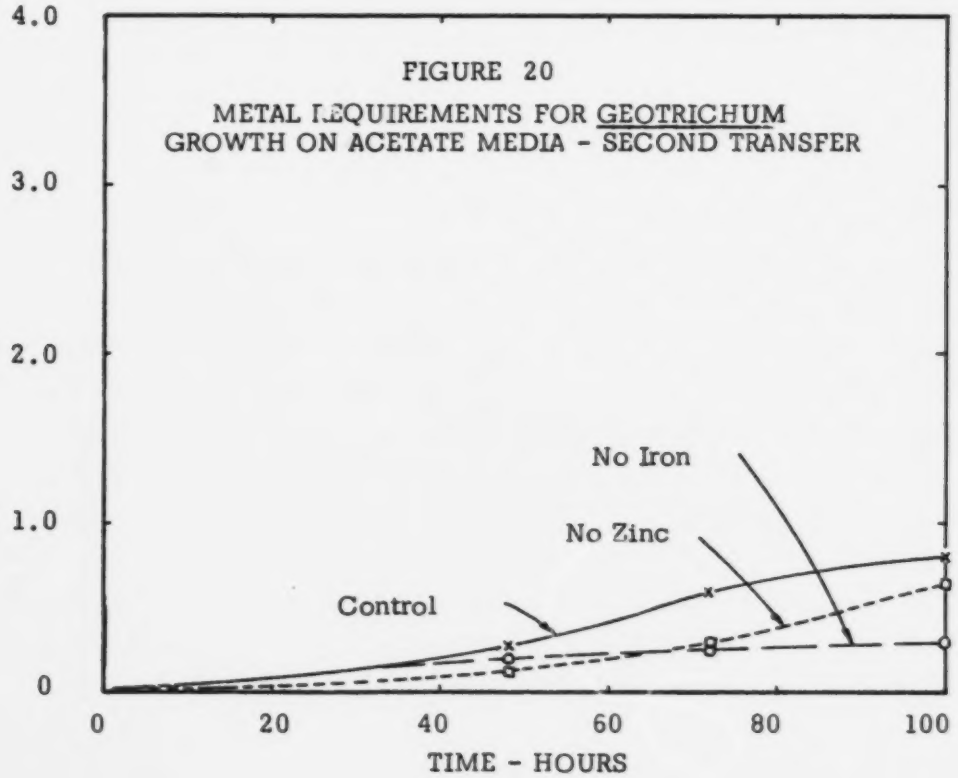


FIGURE 20

METAL REQUIREMENTS FOR GEOTRICHUM GROWTH ON ACETATE MEDIA - SECOND TRANSFER



this fungus. The presence or absence of the other metals did not influence the growth responses. An additional run was mde in which zinc was the only metal added to the media. In 120 hours, the weight of cell mass was 2600 mg/l. This was not as rapid a growth as experienced previously, but only zinc can support a significant growth of this organism in glucose.

Since copper has inhibited growth in other runs, copper was eliminated from the media for the run shown in Figure 24. The growth response was not improved and copper would appear not to inhibit Geotrichum growth on glucose. The need for zinc is evident as shown by the very poor growth in the media without zinc. There also was a reduction in growth due to the lack of iron. This reduction was not highly significant. Since these flasks were seeded by the second dilution technique, there was no carry-over metals from the seed.

9. Growth of Geotrichum on Glycerol Media

Using glycerol as the carbon source, the growth response of Geotrichum to trace metal deficiencies was evaluated. Figure 25 is a plot of the data obtained in the first run in which all flasks were seeded from a common seed flask containing all trace metals. There was good growth in all flasks, excepting the flask without zinc. The growth response on the zinc deficient medium was only about 2/3 of the rest of the media. Since metal carry-over was expected from the seed culture, the second dilution was made with the growth response shown in Figure 26.

Growth was profuse on all but two media. The lack of iron caused an inhibition in the growth. However, the zinc deficiency was most pronounced. Growth was essentially stopped indicating a substantial requirement for zinc in the metabolism of glycerol. While an iron deficiency may retard the Geotrichum growth, a zinc deficiency almost completely inhibits the growth.

Copper inhibition does not appear to exist with this culture and medium. The cultures grown on media without copper produced basically the same growth response as the control. The data shown in Figure 27 were collected to evaluate the effect of copper. The growth rate was slower than those previously attained. This could have been a result of the condition of the seed culture. The maximum growth was normal except in the flasks deficient in iron and in zinc. The medium without zinc essentially did not support any growth.

FIGURE 21

GROWTH OF GEOTRICHUM ON ACETATE MEDIA
COPPER AND COBALT ELIMINATED

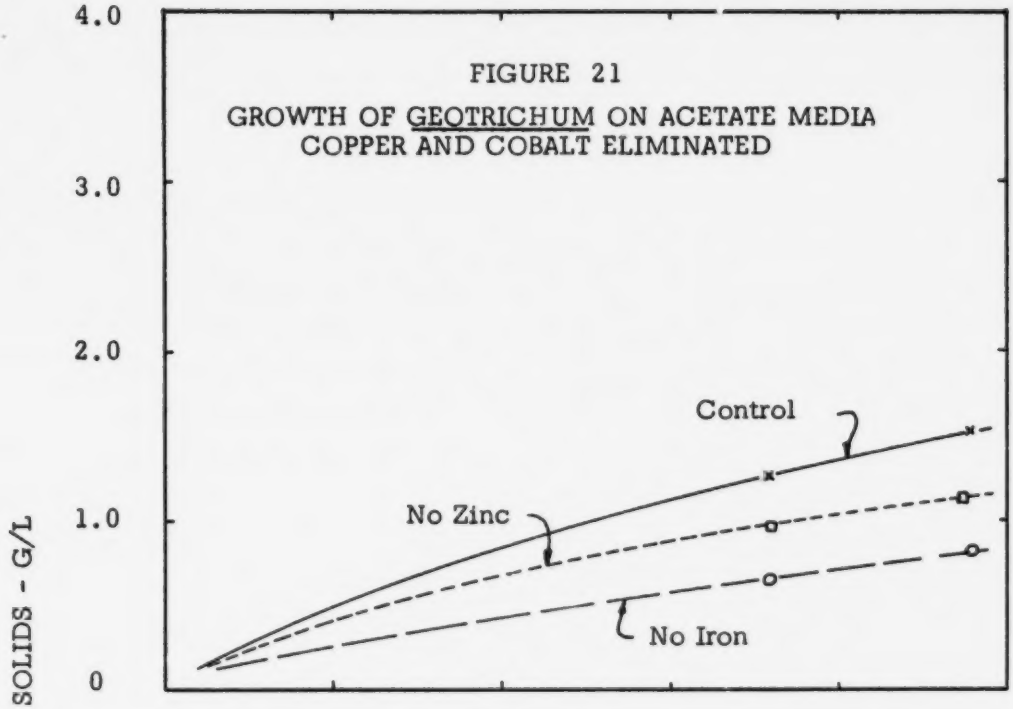
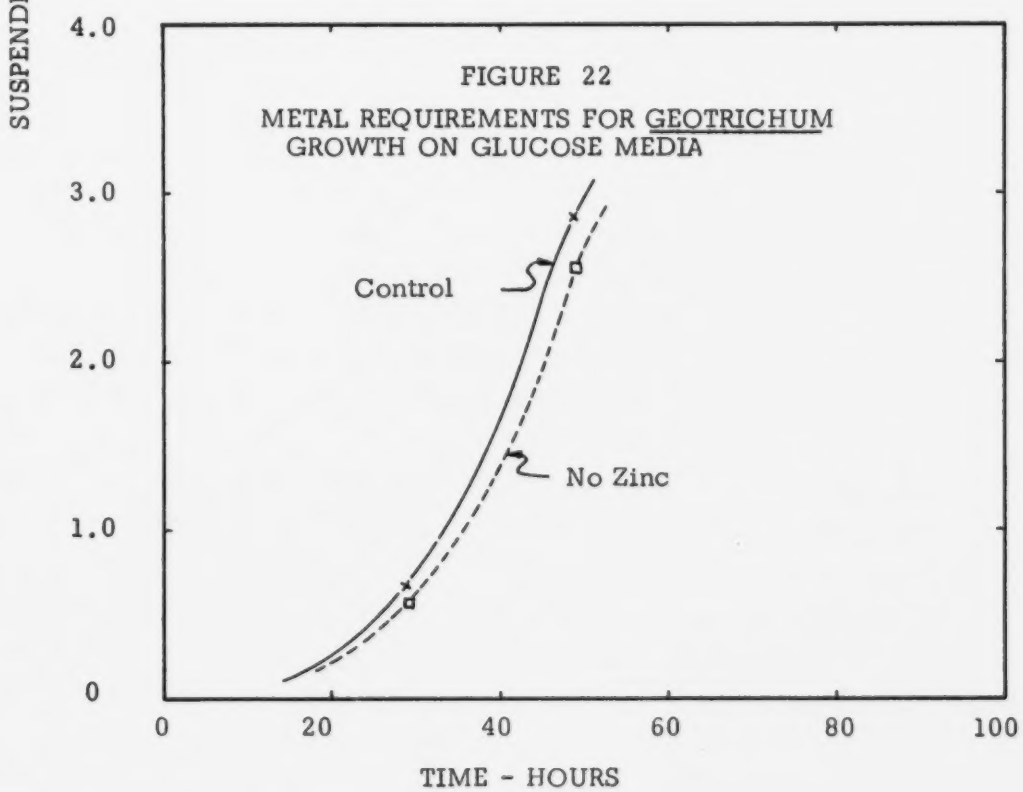


FIGURE 22

METAL REQUIREMENTS FOR GEOTRICHUM
GROWTH ON GLUCOSE MEDIA



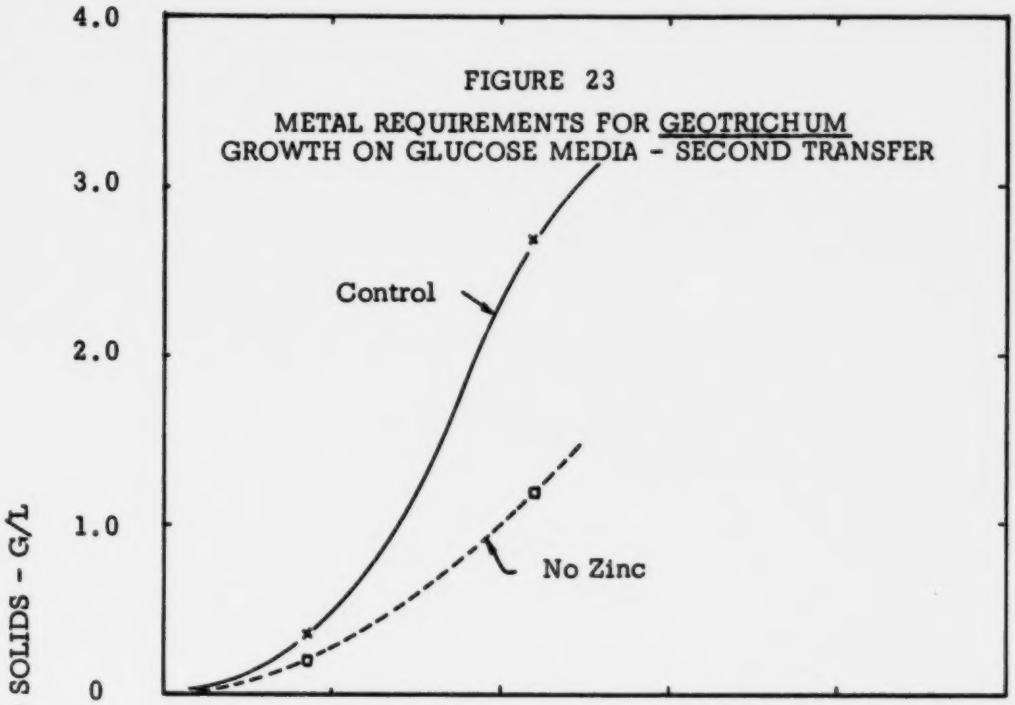


FIGURE 23

METAL REQUIREMENTS FOR GEOTRICHUM
GROWTH ON GLUCOSE MEDIA - SECOND TRANSFER

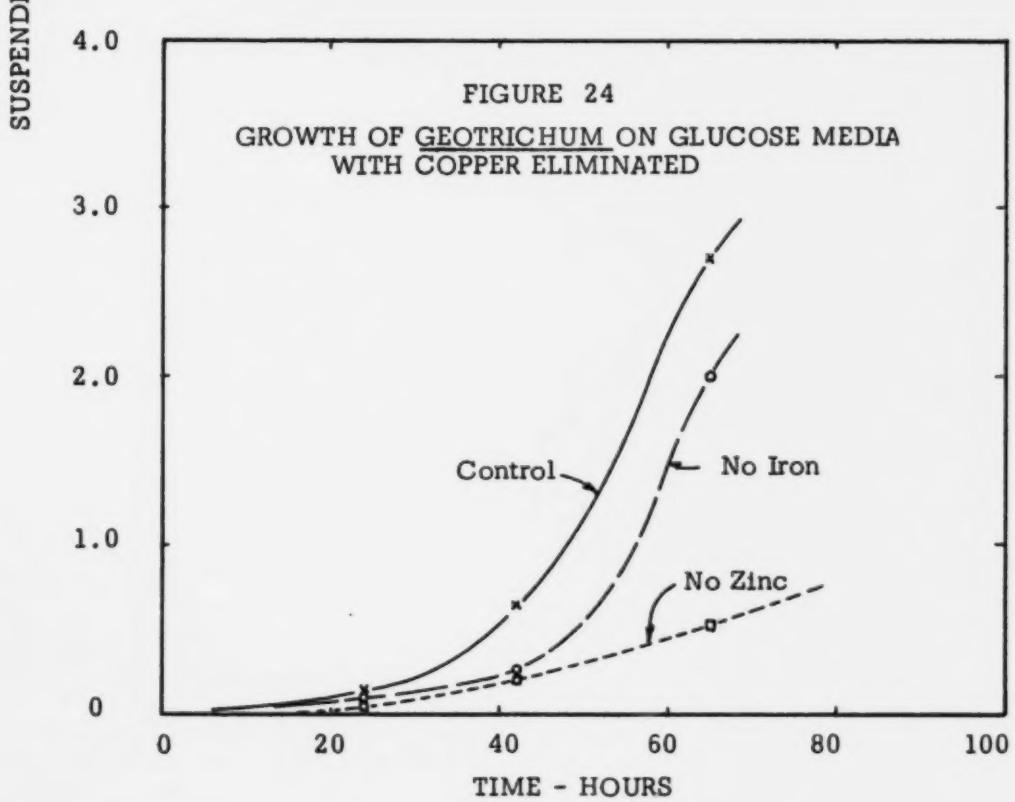


FIGURE 24

GROWTH OF GEOTRICHUM ON GLUCOSE MEDIA
WITH COPPER ELIMINATED

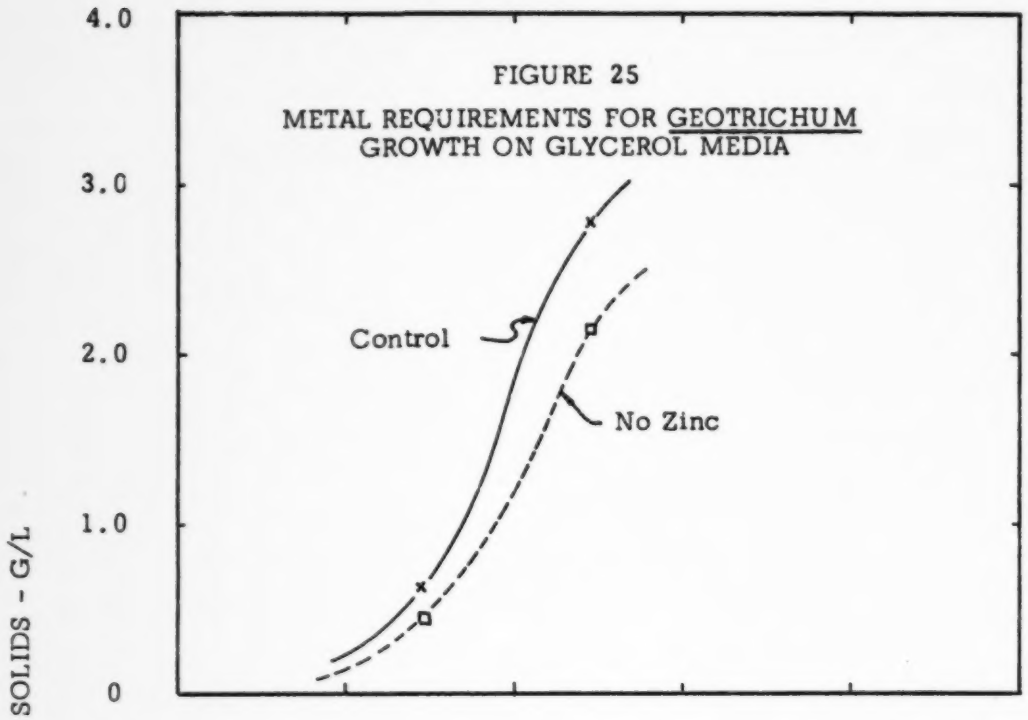


FIGURE 25

METAL REQUIREMENTS FOR GEOTRICHUM GROWTH ON GLYCEROL MEDIA

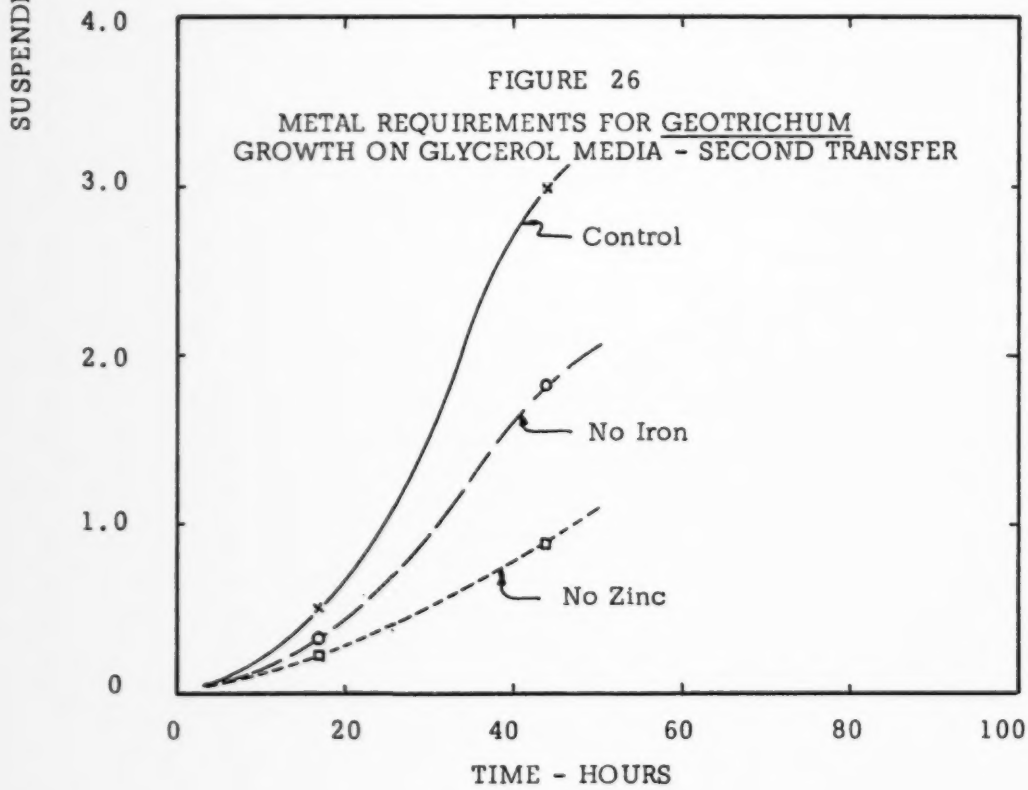


FIGURE 26

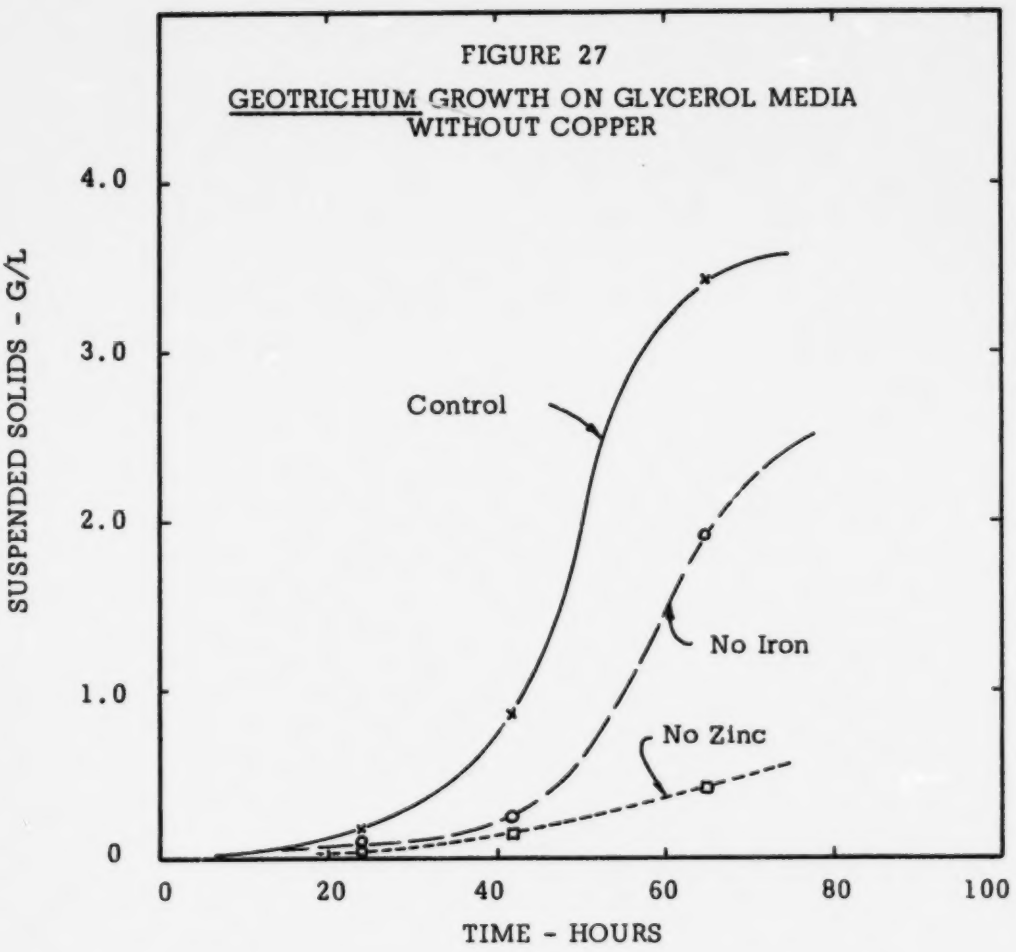
METAL REQUIREMENTS FOR GEOTRICHUM GROWTH ON GLYCEROL MEDIA - SECOND TRANSFER

SUSPENDED SOLIDS - G/L

To ascertain the importance of zinc, the growth response of this organism was measured on a medium containing zinc as the only trace metal. Growth was significant, but retarded. In 5 days, the organism weight was 1800 mg/l. By the sixth day this growth had increased to 2500 mg/l. It would appear that significant growth of Geotrichum was possible on this medium containing zinc as the only trace metal.

FIGURE 27

GEOTRICHUM GROWTH ON GLYCEROL MEDIA WITHOUT COPPER



EXPERIMENTAL RESULTS - PHASE 2

1. Continuous Feed Studies on Glucose Substrate

The initial run was made with glucose as the carbon source. Studies with the pure cultures showed glucose to be preferred over acetate for fungal growth. Combinations of trace metal were selected to ascertain the effect of these combination on the substrate removal and growth characteristics of the mixed culture. The seed of organisms was obtained from domestic sewage. As soon as the mixed liquor solids reached concentrations between 2 to 3 g/l, testing was started by feeding only the desired trace metals.

With a 48 hour liquid retention time, five washouts were obtained in ten days. This would essentially remove all of the components of the original substrate. With a sludge production of 2.5 to 3.0 g/day, the sludge age would be about five to seven days, depending upon the mixed liquor solids concentration. This would mean that 25 to 35 days would be required for washout of the original sludge. Therefore, the results of these studies are not truely representative until adequate time is allowed for not only the liquid washout, but also for the solids washout.

Figure 28 shows the growth response of the mixed culture to glucose substrate containing iron, manganese and zinc as the trace metals. Growth was good on this substrate, with efficient removal of the substrate during the middle of the run. The slightly poorer substrate removal during the latter part of the run was a result of low pH values. It was difficult to maintain the pH level and during this period, the pH was frequently below 6.0. The effluent contained considerable solids as indicated by the high values for the total effluent COD. This was due to the poor settling characteristics of the sludge as evidenced by the SVI values of about 100. Flocculation was excellent with very clear supernatant resulting when the settleable solids test was conducted.

When cobalt was used in place of zinc, the growth response was significantly different. These data are shown in Figure 29. Soluble COD removal was excellent except during the latter part of the run. The pH in this unit was also low during this period. The settleability of the sludge was good having a SVI of about 50. The effluent did contain substantial suspended solids, but this was a result of the characteristics of the settling units. Supernatant in the settling tests was very clear with good flocculation achieved.

FIGURE 28

MIXED CULTURE GROWTH ON GLUCOSE
MEDIA CONTAINING IRON, MANGANESE AND ZINC

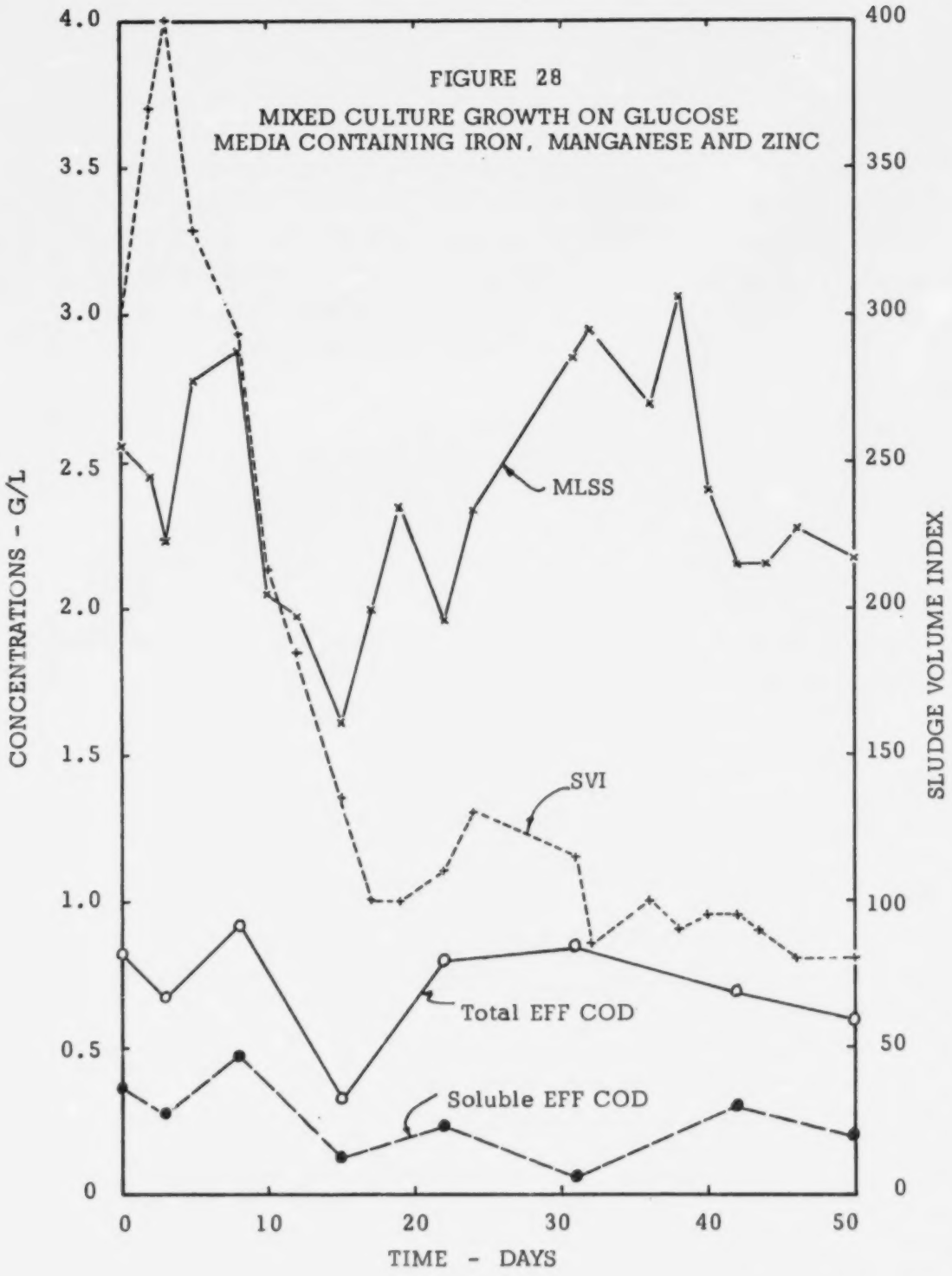
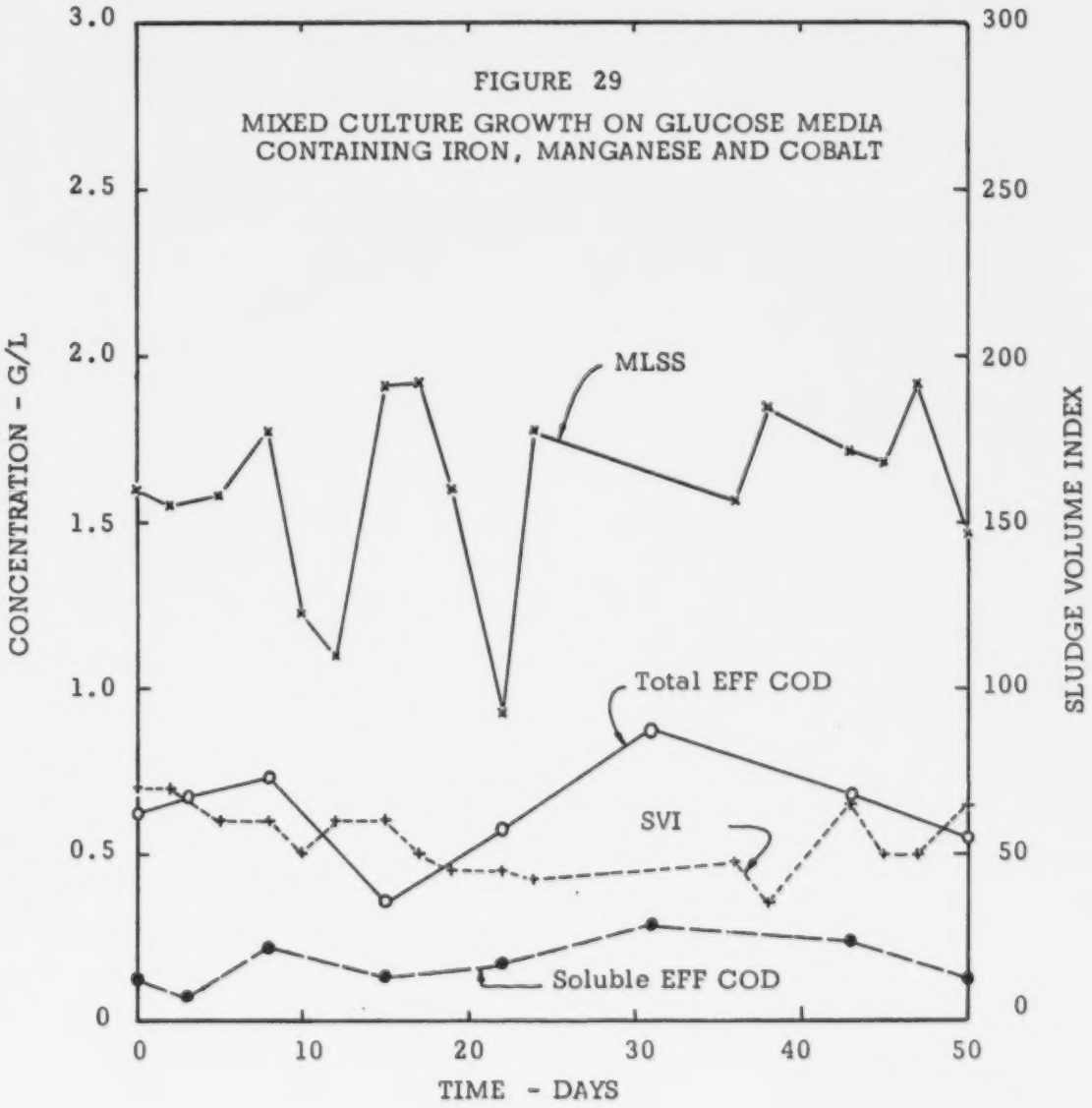


FIGURE 29

MIXED CULTURE GROWTH ON GLUCOSE MEDIA CONTAINING IRON, MANGANESE AND COBALT



When the glucose substrate contained both zinc and cobalt in addition to iron and manganese, the system essentially failed. These data are shown in Figure 30. Substrate removal was very poor. The total COD effluent was high. It was not possible to achieve high mixed liquor solids because the sludge did not flocculate well. The low SVI values were obtained because a substantial portion of the solids did not settle leaving a very turbid supernatant. This combination of metals appeared to inhibit all growths.

Figure 31 shows the growth response of the mixed culture to the glucose substrate containing only iron and zinc. The substrate utilization was efficient with the production of a large mass of sludge. The settling characteristics were very poor with SVI values ranging from 300 to 450. This growth was essentially all fungi. This substrate was an excellent growth medium for this population of organisms , which would not settle adequately.

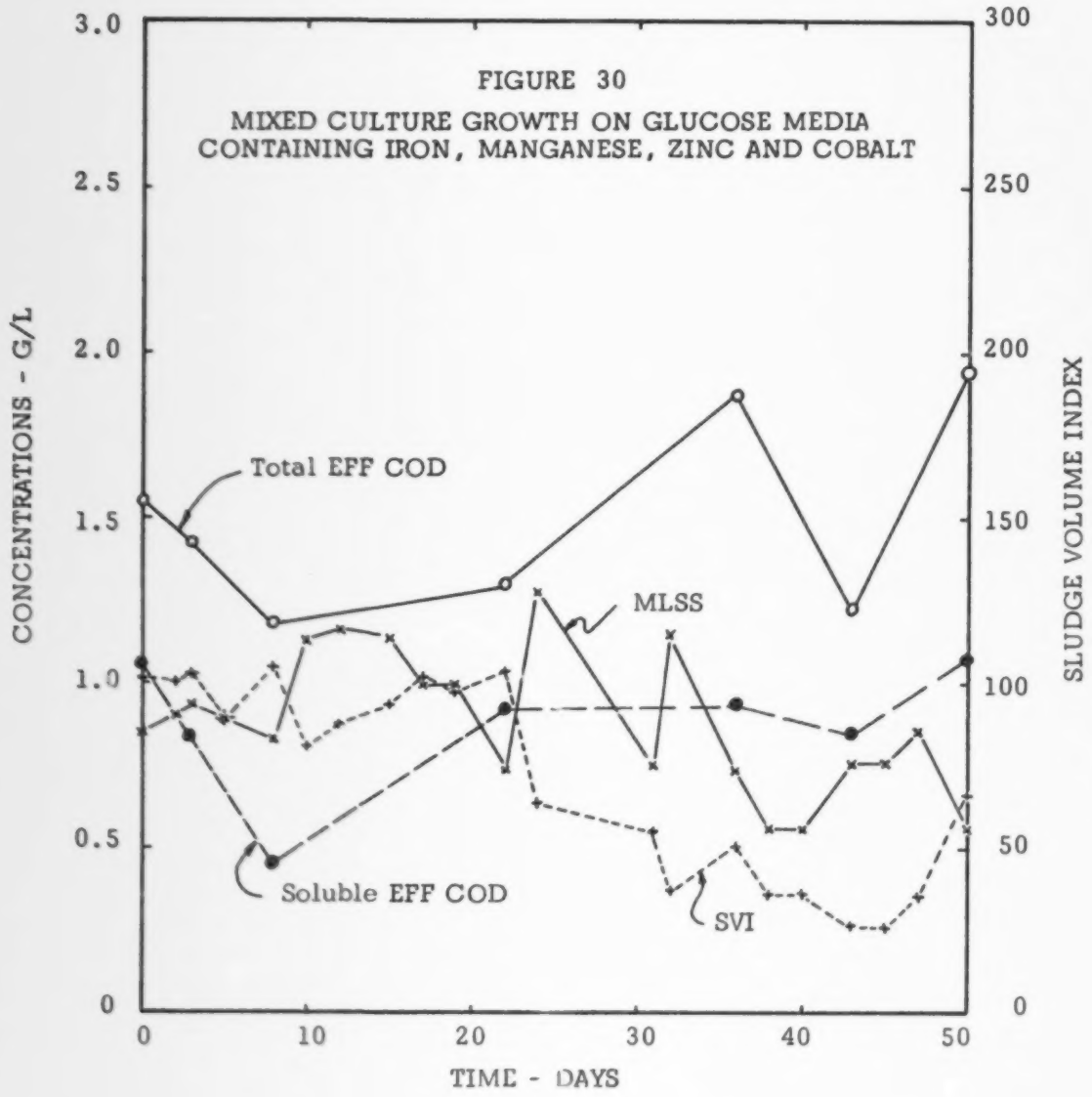
In a glucose medium containing only iron and cobalt, the growth characteristics were undesirable. Figure 32 is a plot of these data . While substrate utilization was adequate, the solids would not flocculate and could not be removed by sedimentation. As a result, it was not possible to maintain a sufficient concentration of mixed liquor solids in the systems. The SVI values were low, but this was caused by the complete lack of sedimentation of part of the sludge. This system appeared to synthesize the substrate, but endogenous respiration was lacking; or at least the characteristics necessary for flocculation were missing. The total effluent COD values were very high as a result of the suspended solids in the effluent.

Growth on glucose medium containing only iron was profuse, but filamentous in nature. Figure 33 shows the results of this test. Good growth was obtained but substrate removal was less than desirable. The resulting sludge had a SVI ranging between 100 and 200. The flocculation characteristics were good. This is expected from a fungal growth. However, it would not settle adequately and the effluent was high in suspended solids.

With the addition of both iron and copper to the glucose substrate, the growth response changed considerably from that of iron alone. These data are shown in Figure 34. Growth was good with efficient utilization of the substrate. The settling characteristics were good with a SVI of 50 or

FIGURE 30

MIXED CULTURE GROWTH ON GLUCOSE MEDIA
CONTAINING IRON, MANGANESE, ZINC AND COBALT



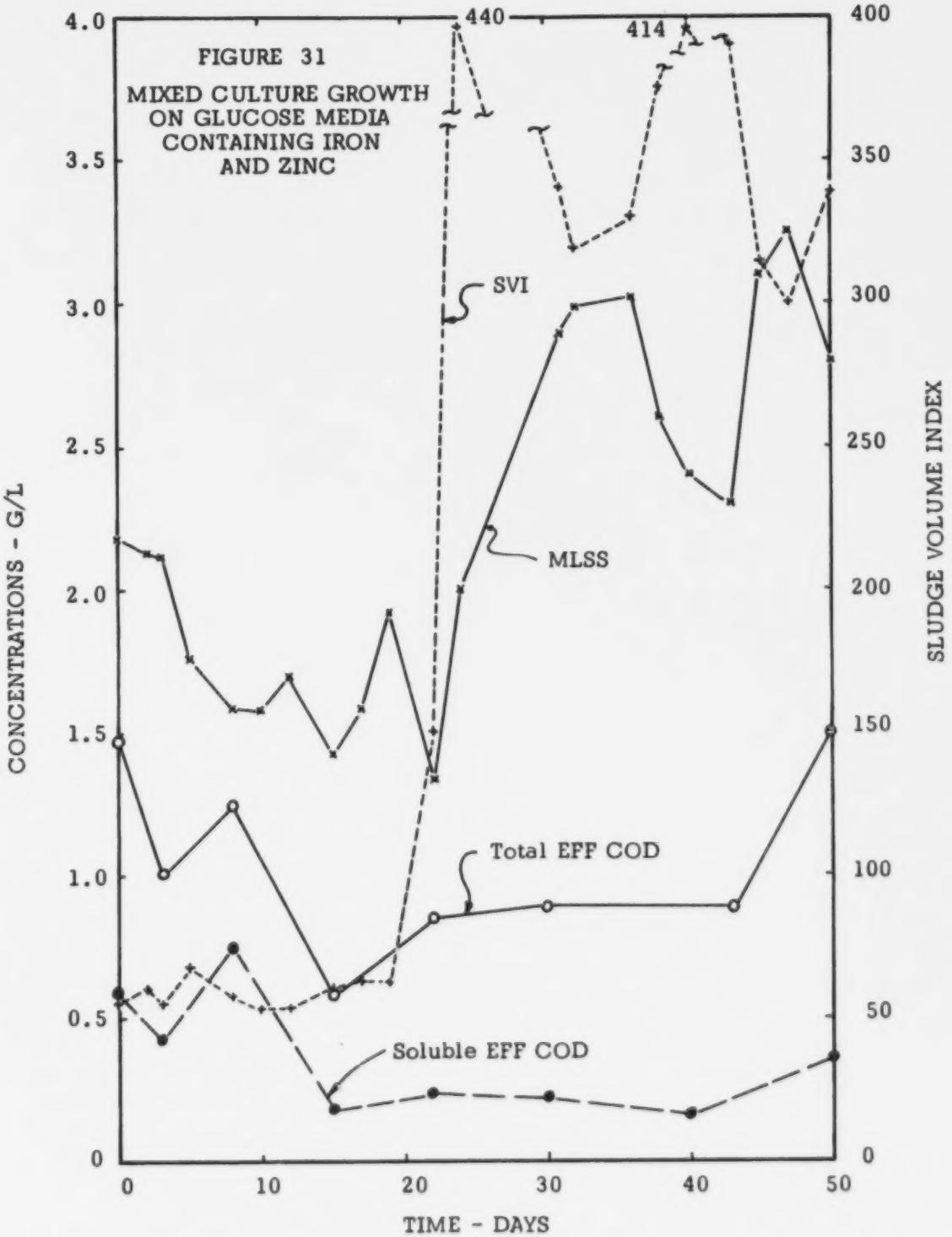
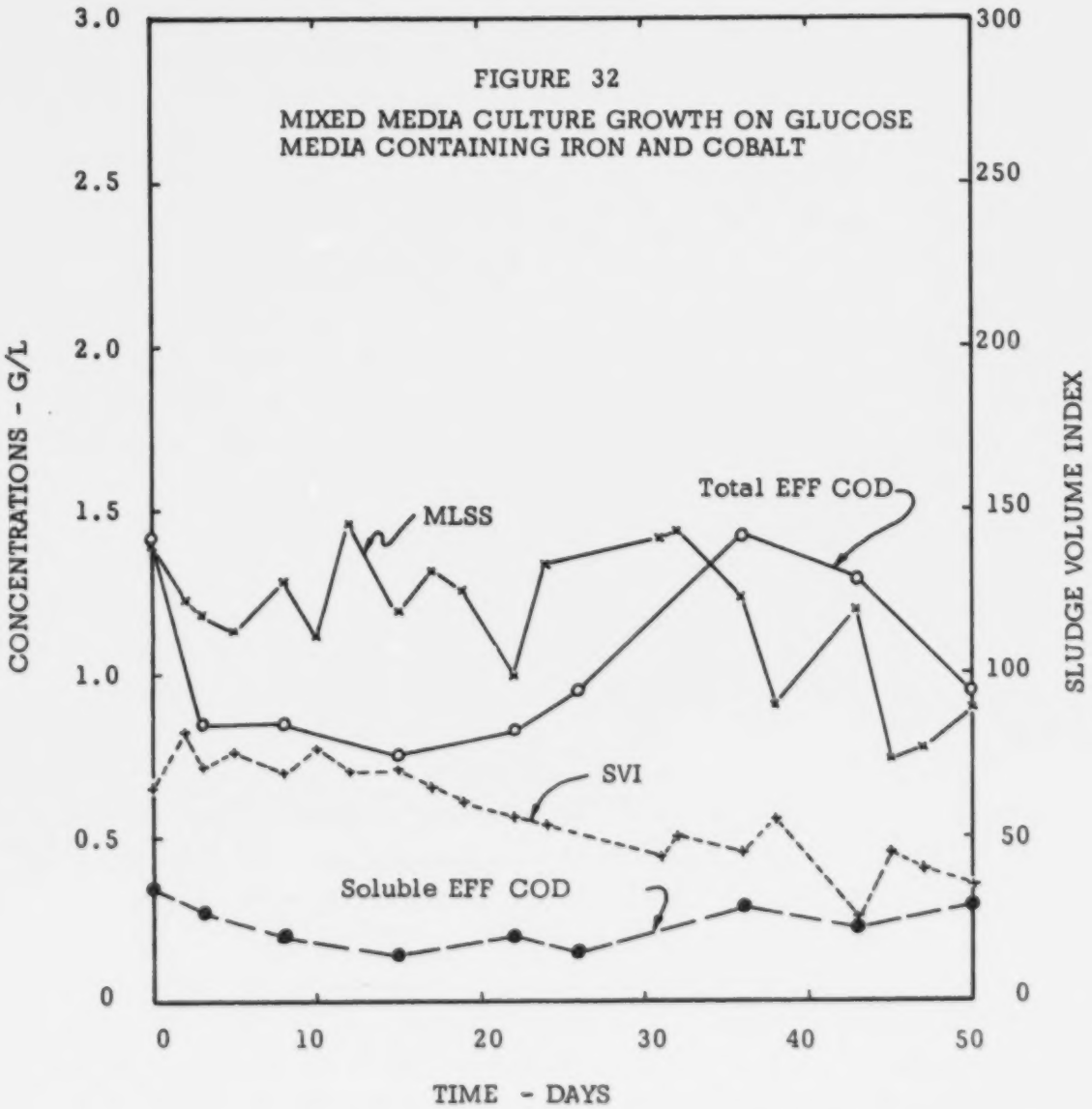


FIGURE 31
MIXED CULTURE GROWTH ON GLUCOSE MEDIA CONTAINING IRON AND ZINC
440
414
SVI
MLSS
Total EFF COD
Soluble EFF COD
CONCENTRATIONS - G/L
SLUDGE VOLUME INDEX
TIME - DAYS
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50

FIGURE 32

MIXED MEDIA CULTURE GROWTH ON GLUCOSE
MEDIA CONTAINING IRON AND COBALT



less. The total COD of the effluent was relatively low and was due primarily to the characteristics of the unit. There was carry-over of solids into the effluent because of the settling tank design. This substrate was the best growth medium for glucose. Bacterial growth was uninhibited and the fungi could not establish any growth.

A rerun on the glucose substrate containing only iron and cobalt was made. These data are shown in Figure 36. The growth response was similar to the first run on this substrate as shown in Figure 32. Substrate utilization at times was adequate. However, the lack of good flocculation of the sludge was evident. It was not possible to maintain the mixed liquor solids at the original level. The sludge that flocculated had a low SVI, generally less than 50. However, only part of the sludge mass was flocculating. A significant amount of the sludge mass was finely dispersed and remained in the effluent.

2. Continuous Feed Studies on Acetate Substrate

A set of experiments was conducted using sodium acetate and acetic acid as the carbon source. The proportion of these two chemicals was varied to maintain the pH in the neutral range. Additional sulfuric acid was added to the units to prevent the pH from increasing. This was necessary because of the different degrees of substrate utilization experienced with the different trace metal combination. This run was conducted in two parts. Initially all of the substrates contained iron with various other trace metals added. The growth response was generally good with all combinations. The iron was then eliminated from all media and the growth response was evaluated.

When iron was the only trace metal added to the basic substrate, the growth response was very good. These data are shown in Table 14. Substrate utilization was complete producing a sludge with SVI values ranging between 50 and 75. This appeared to be a satisfactory medium for desirable mixed culture growth.

Since iron was the only trace metal in the substrate, it should be responsible for good growth. To check this assumption, iron was eliminated from the substrate. The system was operated for about 30 days before data were collected. These results are shown in Figure 37. The growth

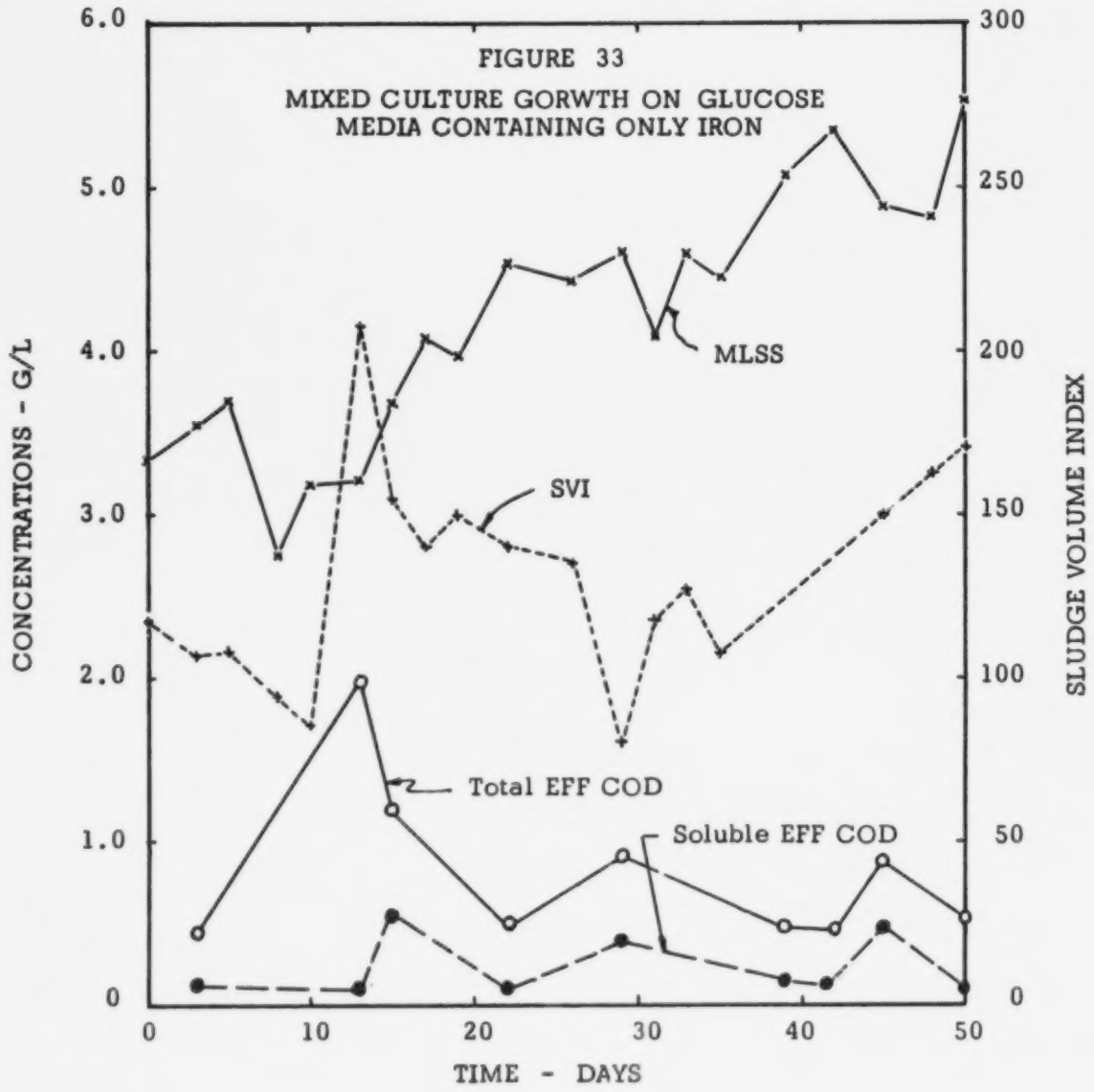
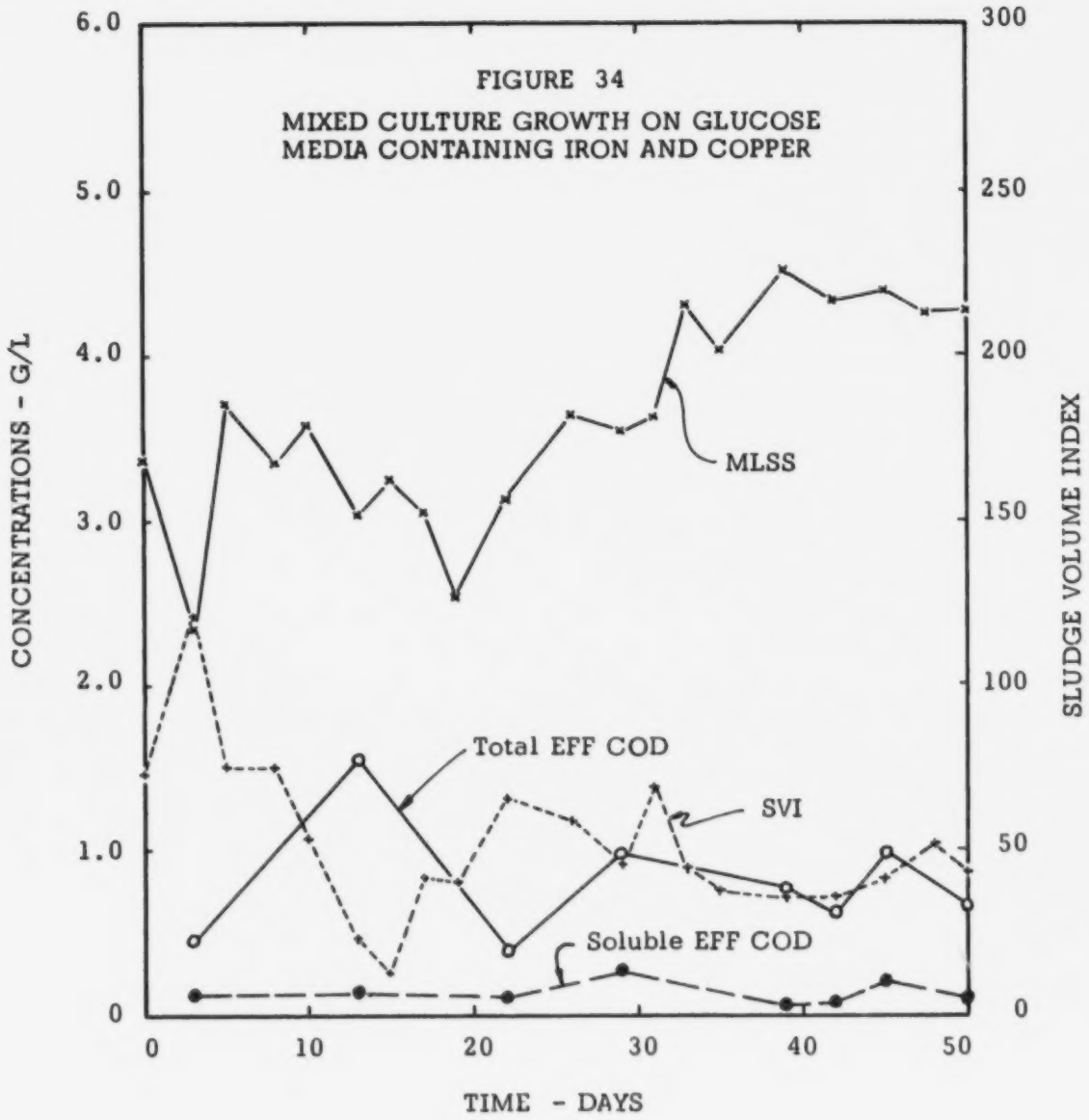


FIGURE 33

MIXED CULTURE GORWTH ON GLUCOSE MEDIA CONTAINING ONLY IRON

FIGURE 34

MIXED CULTURE GROWTH ON GLUCOSE
MEDIA CONTAINING IRON AND COPPER



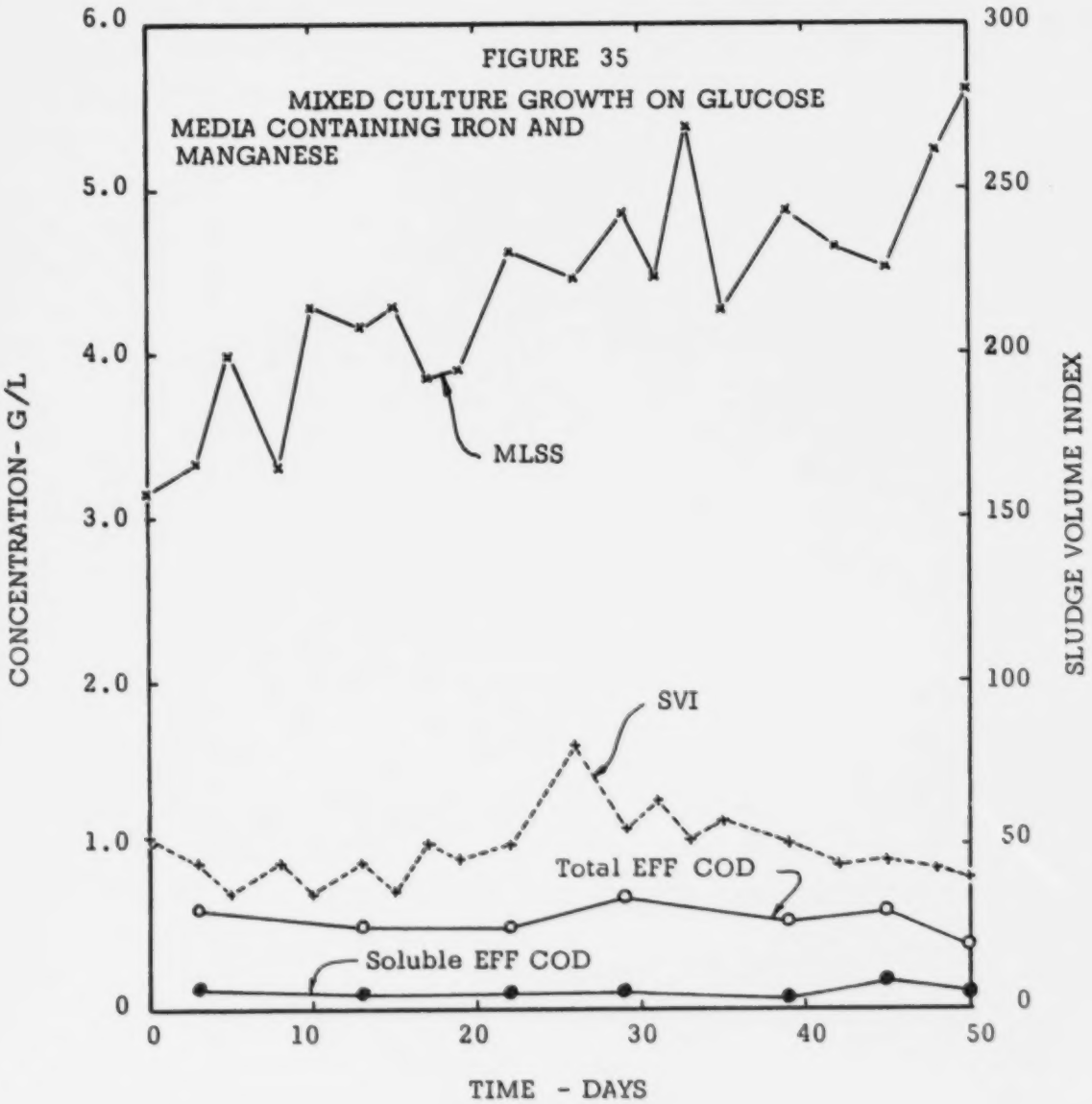


FIGURE 35

MIXED CULTURE GROWTH ON GLUCOSE MEDIA CONTAINING IRON AND MANGANESE

FIGURE 36

MIXED CULTURE GROWTH ON GLUCOSE MEDIA CONTAINING IRON AND COBALT

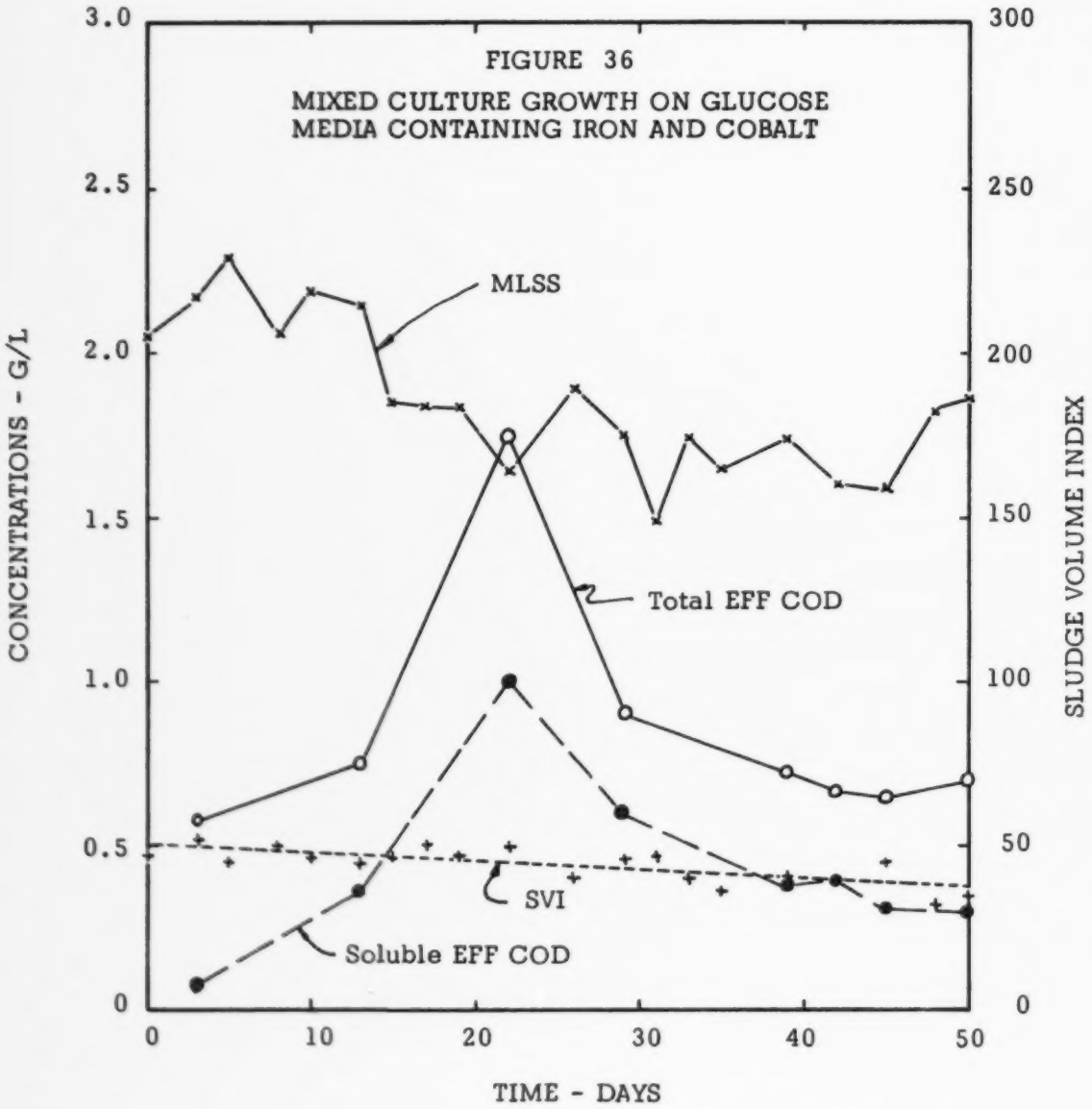


TABLE 14

MIXED CULTURE GROWTH ON ACETATE MEDIA CONTAINING ONLY IRON

Time Days	MLSS Mg/l	SVI	Effluent COD - Mg/l Total	Soluble
0	3400	55	-	-
7	3000	65	350	42
11	2800	60	-	-
14	3100	55	415	37
24	2600	60	-	-
29	2700	50	336	32
36	2600	70	182	51
40	2600	75	-	-
44	3000	50	-	-

characteristics for the first 10 days were very good. Because of the high mixed liquor suspended solids, it was felt that there was sufficient iron in these solids to sustain the growth. Solids were wasted from the unit at this time to reduce the mixed liquor solids from about 4000 mg/l to about 1500 mg/l.

As a result of this removal of the solids, the growth of the organisms gradually deteriorated. The substrate utilization was reduced substantially. The mixed liquor suspended solids continued to decrease and with eventual failure of the system. These data indicate the necessity of iron for growth of any organisms on the acetate substrate.

When only iron and manganese are present in the acetate substrate, growth is excellent with complete substrate utilization. The sludge tends toward filamentous growth with SVI values of about 100. Then data are presented in Table 15.

When the iron was removed from the substrate, the growth continued, producing an ever more filamentous sludge. After about 30 days of operation, data collection was initiated showing excellent substrate utilization as well as very good flocculation (see Figure 38). The SVI was in excess of 200. To ascertain the effect of the iron on the sludge solids, the mixed liquor solids were wasted at the end of the first week to reduce the solids level

FIGURE 37
MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING NO METAL ADDITIVES

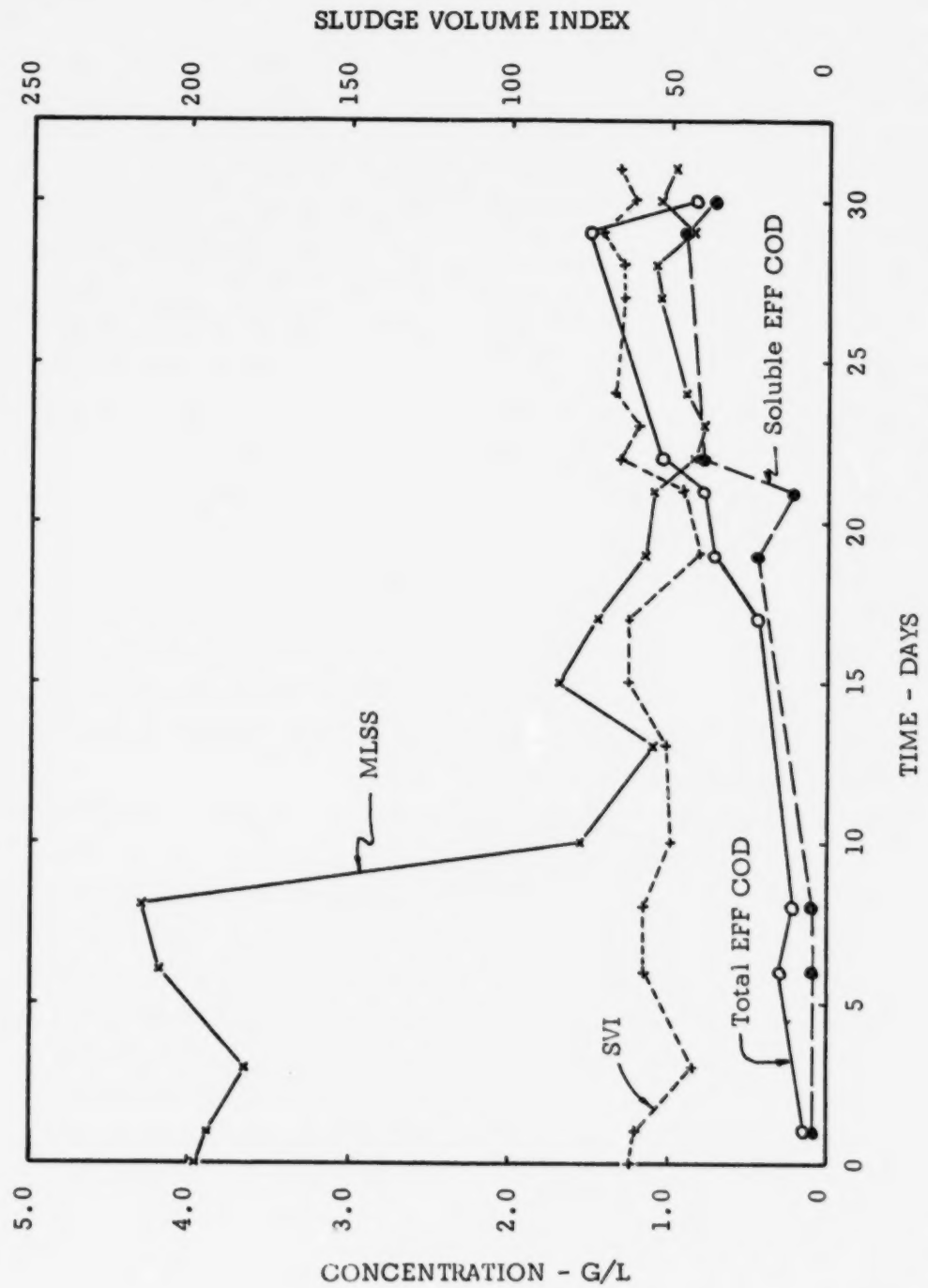


TABLE 15

MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING IRON AND MANGANESE

Time Days	MLSS mg/l	SVI	Effluent COD - mg/l Total	Soluble
0	3800	70	-	-
7	3400	80	570	61
11	3400	85	-	-
14	3700	75	556	45
24	3900	85	-	-
29	-	-	350	35
36	4000	100	623	68
40	3900	115	-	-
44	4100	100	-	-

from about 2500 mg/l to about 1250 mg/l. By the end of the following week, the mixed liquor solids had again increased to 2000 mg/l. The sludge was again wasted. This had no effect on the growth and by the end of the run, the mixed liquor solids had again increased to 2000 mg/l.

During the entire run, substrate utilization was very good. The sludge flocculated very well and the solids concentration in the effluent was very low. With the wasting of sludge, the SVI was maintained at about 100. This was indicative of slight bulking growth. It would appear that manganese can substitute for iron in the trace metal requirements for acetate metabolism.

Acetate substrate containing iron, manganese and zinc produced good growth characteristics. Substrate utilization was complete and the sludge had a SVI that was increasing with time, and possibly could have resulted in a poor settling sludge. These data are shown in Table 16. The run was not continued and this possibility was not evaluated.

When the iron was eliminated from the substrate, the SVI had increased to 150 by the time data collection began. However, upon wasting the sludge solids to reduce the mixed liquor suspended solids from 3500 mg/l to about 1500 mg/l, the SVI decreased to below 100 and remained between 50 and 100 for the remainder of the run. These data are shown in

FIGURE 38

MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING ONLY MANGANESE

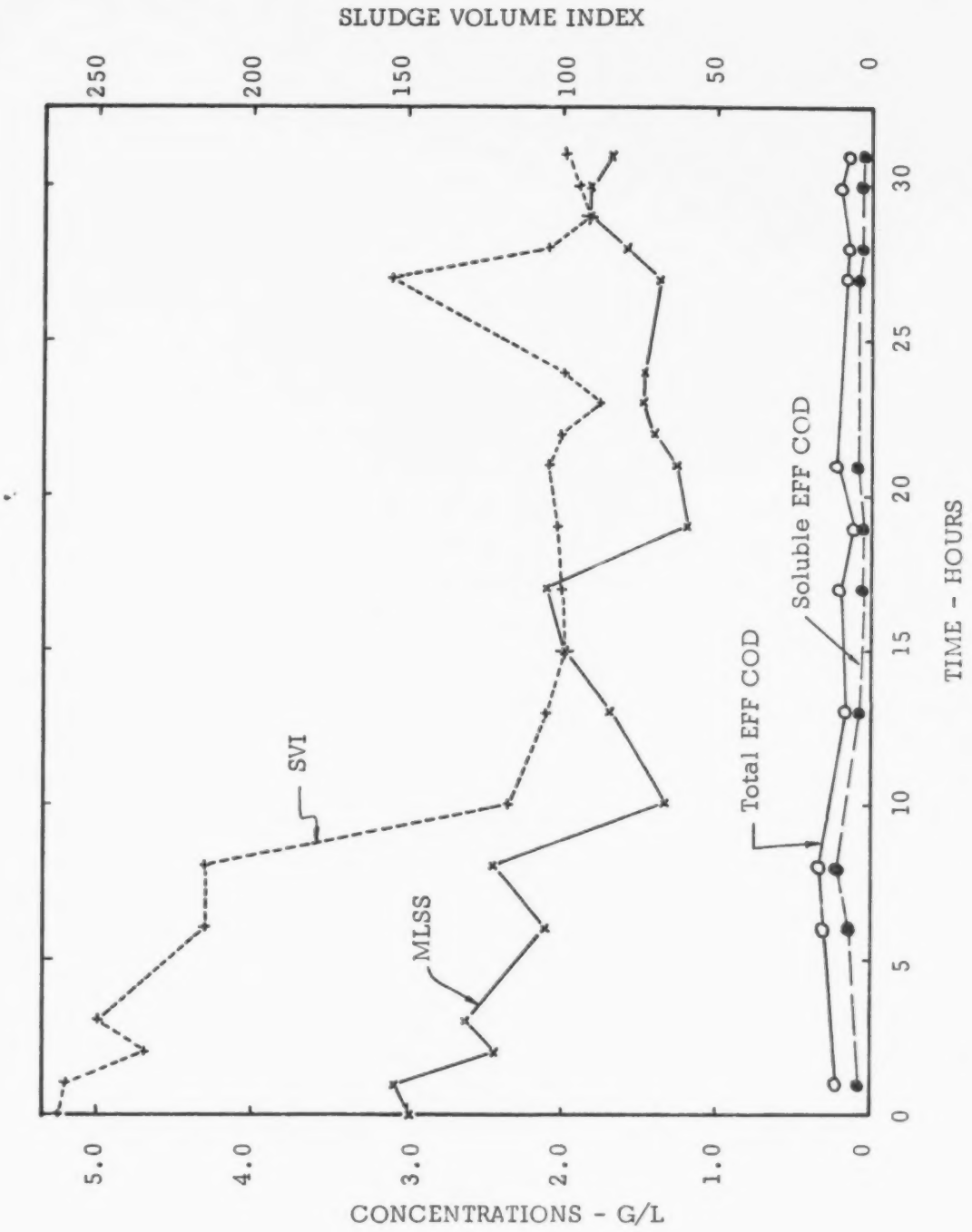


TABLE 16

MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING IRON, MANGANESE AND ZINC

Time Days	MLSS mg/l	SVI	Effluent COD - mg/l Total	Soluble
0	3600	60	-	-
7	2900	60	390	49
11	2800	60	-	-
14	2800	55	378	40
24	2800	60	-	-
29	2500	65	458	46
36	2600	80	370	51
40	2300	95	-	-
44	2500	90	-	-

Figure 39. The substrate utilization was very good throughout the entire run. After the sludge was wasted, the mixed liquor solids began to increase and at the end of the run had a concentration of about 2200 mg/l. The growth response to this substrate was good and the deletion of the iron or addition of zinc had no apparent effect on the microbial growth.

Cobalt was added to the acetate substrate containing iron, manganese and zinc. The growth response is given in Table 17. With the cobalt, the characteristics of the sludge was changed. While substrate utilization was

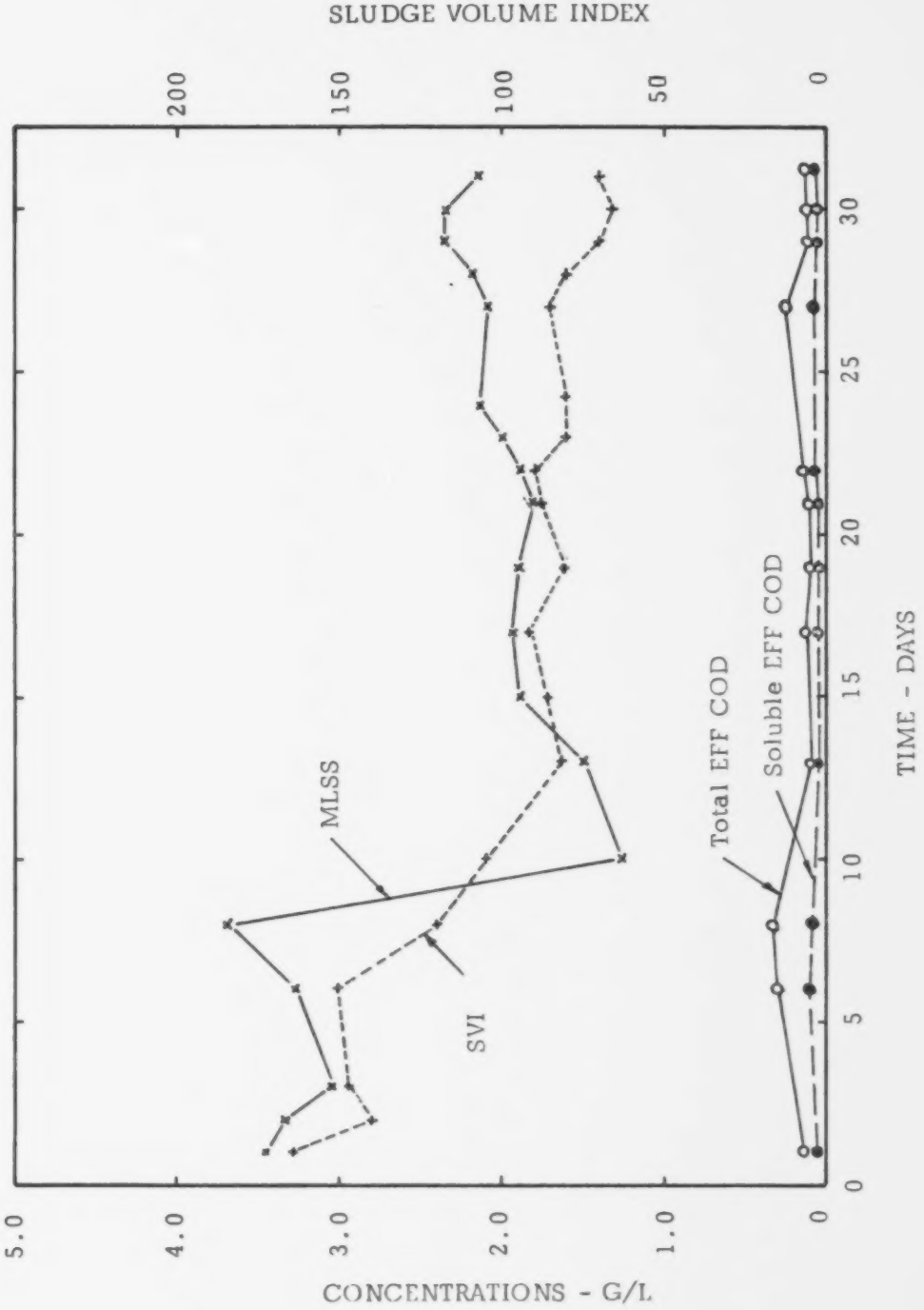
TABLE 17

MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING IRON, MANGANESE, ZINC AND COBALT

Time Day	MLSS mg/l	SVI	Effluent COD - mg/l Total	Soluble
0	4000	55	-	-
7	3100	65	150	57
11	2800	70	-	-
14	3000	60	490	30
24	2800	60	-	-
29	2400	55	450	46
36	2300	60	560	58
44	2200	55	-	-

FIGURE 39

MIXED CULTURE GROWTH ON ACETATE MEDIA
CONTAINING MANGANESE AND ZINC



complete, and the SVI was low, there was a dispersed growth present that did not flocculate. This resulted in a decrease in mixed liquor solids. The total effluent COD was about 500 mg/l greater than the soluble COD. This was caused by the suspended solids in the effluent. These solids were not all a result of the characteristics of the settling tanks, but partially resulted from this dispersed growth. The supernatant from settling tests was very turbid.

Elimination of the iron did not improve the growth response. The effluent was very turbid as a result of the dispersed growth. The substrate utilization decreased substantially with soluble effluent COD values as high as 500 mg/l. These data are shown in Figure 40. The mixed liquor suspended solids did not increase even though SVI values less than 50 were measured. It would appear that the cobalt was inhibiting the growth of the mixed culture.

A similar situation existed when the acetate substrate contained iron, manganese, zinc, cobalt and copper. These data are given in Table 18. Substrate utilization was good and sludge that settled had a low SVI. Again, though, the settling was not complete as there was substantial dispersal growth.

TABLE 18

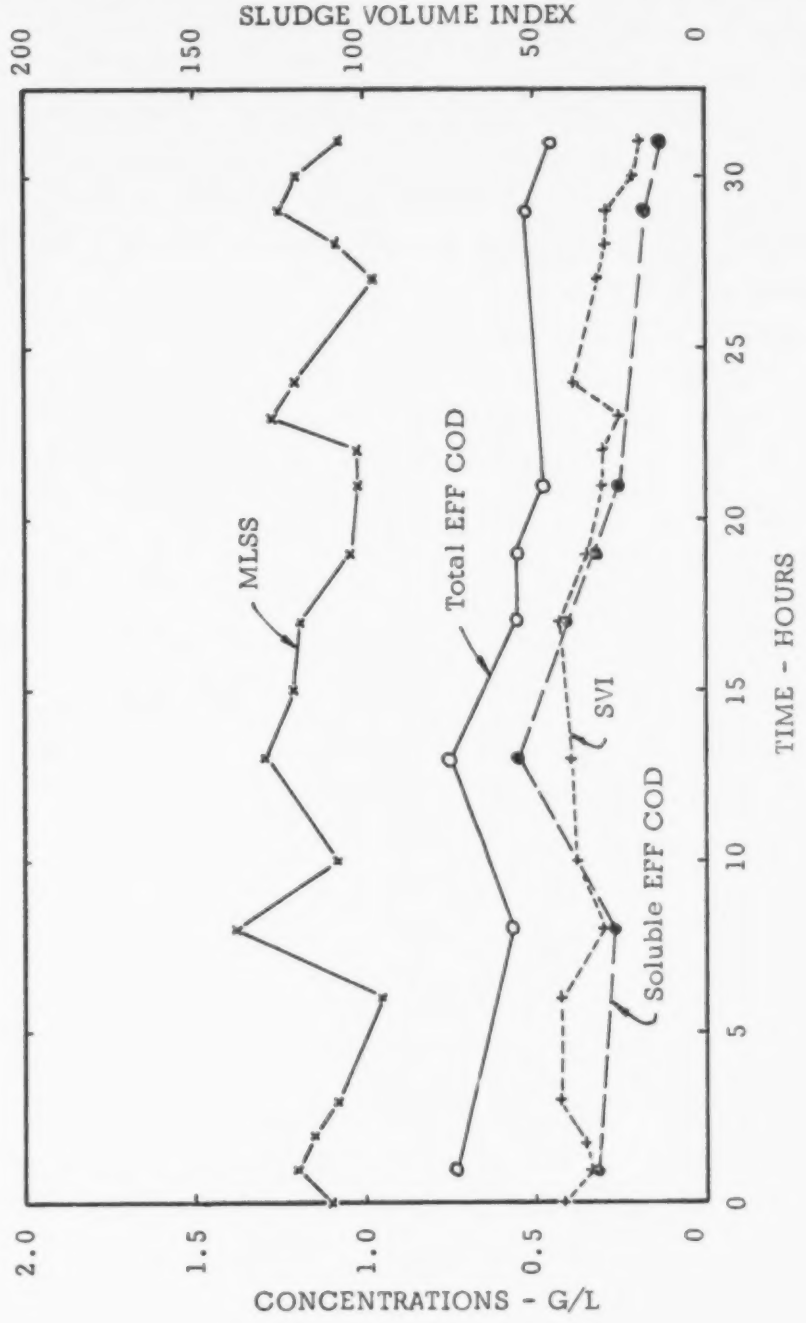
MIXED CULTURE GROWTH ON ACETATE
MEDIA CONTAINING IRON, MANGANESE, ZINC, COBALT AND COPPER

Time Days	MLSS mg/l	SVI	Effluent COD- mg/l	
			Total	Soluble
0	3800	55	-	-
7	3200	60	964	46
11	3200	55	-	-
14	3100	50	519	52
24	2600	40	-	-
29	2100	50	293	50
36	2300	55	466	65
44	2800	65	-	-

When the iron was eliminated, the soluble COD in the effluent increased indicating incomplete substrate utilization. These data are shown in Figure 41. Wasting of the mixed liquor solids to decrease the concentration

FIGURE 40

MIXED CULTURE GROWTH ON ACETATE MEDIA
CONTAINING MANGANESE, ZINC AND COBALT

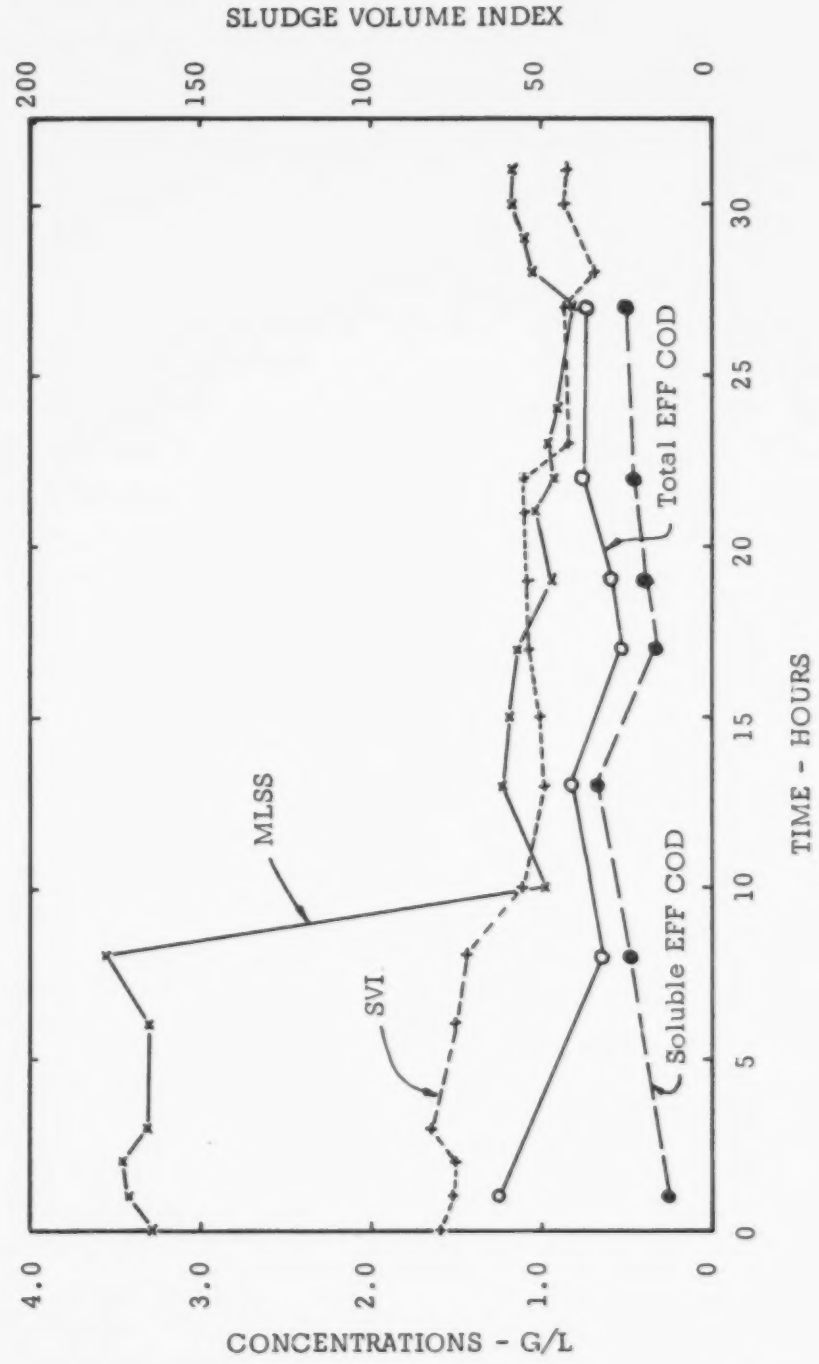


from about 3500 mg/l to 1000 mg/l caused virtual failure of the process. The solids concentration never increased above the 1000 mg/l. The addition of the copper did not improve the growth of the mixed culture on this particular substrate.

There was a general inhibition of growth in these systems containing cobalt and copper. The effect was enhanced when iron was missing. The growth was not filamentous, but exhibited poor flocculating characteristics. While substrate utilization may have been adequate, the desired efficiency could not be obtained because of the inability to separate the sludge solids.

FIGURE 41

MIXED CULTURE GROWTH ON ACETATE MEDIA
CONTAINING MANGANESE, ZINC, COBALT AND COPPER



DISCUSSION OF RESULTS

1. Preparation of Synthetic Media

Extreme care must be exercised in the preparation of the chemically defined medium. When chemicals are added to the water, many hydrolytic reactions occur. In particular, the formation of metal hydroxides by such metals as iron, zinc, and manganese can cause these metals to precipitate to such a low level that they are not available as trace nutrients. The ability of these metals to form soluble complexes with various amine groups as well as other organic compounds, will maintain them soluble as long as these organic materials are available for complex formation.

Iron is the most critical in the precipitation reaction. Use of ferrous iron salts and sterilization by filtration rather than heat will reduce the rate of precipitation. Addition of the iron to the total media and then heat sterlizing will render it unsoluble. If this iron is the only source, it may well be the rate limiting nutrient. Of course, this problem primarily exists when simple chemicals are the basis for the substrate composition.

An even more critical situation can exist when the phosphate buffer system is used for pH control. The phosphate salts of most metals are very insoluble. When cations such as calcium, iron, zinc, etc. are autoclaved with the high concentration of phosphate ion species used for pH control, precipitation reactions are nearly complete, causing loss of a number of these nutrients. Growth on this type of substrate is extremely slow and does not represent the true growth response of the microorganisms to the test conditions. The growth is limited by the availability of the metal ion.

From examination of the literature dealing with studies of growth of microorganisms on chemically defined substrates, it would appear that much of the work has suffered from this problem. Many of the workers employed phosphate as the buffer system failing to recognize the chemical reactions that can occur. The growth limiting factor would be the very minute concentrations of metal nutrients, rather than the particular test condition. This will be true as long as the substrate did not contain a complexing agent which maintains the metals in solution by forming metal complexes.

Use of the bicarbonate buffer system will eliminate the phosphate precipitation. However, pH control is a little more difficult because the bicarbonate system does not regulate the pH as well as the phosphate

system. When the studies are being conducted with substrates containing irorganic nutrients plus a simple carbon source, it will be necessary to use the bicarbonate system. An alternate is to add some organic, such as amines, that can complex the metals and maintain them in solution.

2. Calcium and Magnesium Requirements of Fungi

These tests were inconclusive as a result of the limited number conducted. In general, it would appear that the calcium was not essential in the growth of Fusarium on any of the media tested. Good growth was obtained at zero calcium when magnesium was present. However, it was not possible to obtain any growth without at least one of these ions present. Calcium concentration of 50 mg/l substantially improved the growth of Fusarium on the glucose medium. This would indicate improved fungal growth in hard water regions.

The presence of magnesium was important. When the magnesium concentration was zero, Fusarium growth was inhibited on all media tested. Concentrations of 10 mg/l were sufficient to produce good growth of this fungi on glucose. It also appeared that higher concentrations did not improve the fungal growth. Since the basic media proposed in the literature contained high magnesium, this was not changed. Because of the calcium and phosphate precipitation, the calcium was reduced to 20 mg/l. These concentrations did not inhibit the growth of the fungi as indicated by these limited tests.

3. Trace Metal Requirements for Acetate Metabolism by Fungi

Fusarium grew very well on the acetate medium as long as iron and zinc were present. The presence or absence of the additional trace metals did not influence the growth response. Iron was the more critical, its absence retarding the growth more than the absence of zinc. The only trace metal required in the basic acetate medium was iron. This produced reasonably good growth. When zinc was present with the iron, the growth was very good.

The growth of Geotrichum on acetate was relatively poor under all conditions. The presence of copper and cobalt in this medium did not improve growth, but appeared to inhibit it. The only metal that was definitely required was iron. The presence or absence of zinc, manganese or molybdenum did not influence the growth significantly.

The results of the studies on the acetate medium show that only iron is a critical trace metal. As long as this metal is present along with the major nutrients, growth of fungi can be expected. The poor growth of Geotrichum is a genus characteristic. With the excellent growth of Fusarium, it would be expected that there are other fungi having similar physiological characteristics that may be expected to exhibit these same growth responses.

4. Trace Metal Requirements for Glucose Metabolism by Fungi

Glucose proved to be an excellent carbon source for both species of fungi. This would be expected. When copper was not used in the medium, growth of Fusarium was substantially reduced by the absence of iron or zinc. These metals appeared interchangeable as the absence of either metal had the same growth inhibition. The addition of copper to the media inhibited Fusarium growth. The absence of iron appeared to counteract the copper effect, but the significance of this effect is questionable.

Glucose supported an excellent growth of Geotrichum. The only trace metal required was zinc. It was possible to obtain significant growth of this fungus on glucose media containing zinc as the only trace metal. Copper was not influential in the growth response of this organism. Growth was independent of the presence or absence of copper in the media.

The growth of these two fungi on glucose was primarily controlled by zinc. This would appear to be the only trace metal of importance since growth can be obtained with only this metal present. However, the presence of both iron and zinc in the medium did stimulate the growth of Fusarium. This same stimulation may have also been exhibited by Geotrichum. However, the growth response to iron deficiency was not reduced sufficiently to be highly significant.

5. Trace Metal Requirements for Metabolism of Glycerol by Fungi

The presence of copper in this medium inhibited the growth of Fusarium. Again there appeared to be an interaction in the inhibiting effect of copper with the presence of iron. When iron was absent from the media, the copper effect was reduced. Elimination of copper from the media produced excellent growth with iron as the only essential trace metal.

Copper inhibition of Geotrichum on this medium did not exist. Excellent growth was obtained with copper in the substrate. The absence of zinc severely retarded the growth. Significant growth was obtained on substrate

containing zinc as the only trace metal. Iron deficiency did reduce the growth response, but not nearly as much as the zinc deficiency.

The growth of these two organisms on this medium was controlled by iron and zinc. Copper inhibition of the growth of Fusarium existed. Geotrichum growth was independent of the copper. It would appear that copper inhibited the growth of fungi only on carbon sources that were not the optimum.

6. Summary of Trace Metal Requirements for Fungal Growth

Table 19 is a summary of the trace metal requirements of the two genera of fungi tested. Iron and zinc were the only metals required to obtain good growth of these organisms on appropriate carbon sources. Fusarium growth was excellent on acetate, but only fair on both glucose and glycerol.

TABLE 19

SUMMARY OF TRACE METAL REQUIREMENTS FOR FUSARIUM AND GEOTRICHUM GROWTH

Culture	Carbon Source	Growth Response	Primary Metal	Secondary Metal	Copper Inhibition
Fusarium	Acetate	Good	Iron	Zinc	Absent
	Glucose	Fair	Iron, Zinc	---	Present
	Glycerol	Fair	Iron	None	Present
Geotrichum	Acetate	Poor	Iron	None	Present
	Glucose	Good	Zinc	Iron	Absent
	Glycerol	Good	Zinc	Iron	Absent

Geotrichum exhibited excellent growth on glucose and glycerol, but poor growth on acetate. Copper inhibition was present whenever the growth response to the carbon source was not excellent. This copper effect was apparently related to the overall growth response of the organism.

7. Mixed Culture Growth on Glucose and Acetate Substrate

Table 20 is a qualitative summary of the growth of the mixed cultures on glucose substrate containing various combinations of metals. Growth and substrate utilization are used to indicate the conversion of the substrate to cell mass. The SVI and sludge flocculation are characteristics used to evaluate the ability to remove the synthesized sludge from the effluent.

TABLE 20

MIXED CULTURE GROWTH ON GLUCOSE SUBSTRATE

Metals Added	Growth	Substrate Utilization	SVI	Sludge Flocculation
Fe, Mn, Zn	Excellent	95%	80 - 100	Excellent
Fe, Mn, Co	Good	90%	50	Excellent
Fe, Mn, Zn, Co	Poor	50%	30 - 50	Poor
Fe, Zn	Good	90%	300 - 450	Excellent
Fe, Co	Poor	85%	30 - 50	Poor
Fe	Good	85%	100 - 200	Excellent
Fe, Cu	Good	95%	40 - 60	Poor
Fe, Mn	Excellent	+95%	50	Excellent
Fe, Co	Poor	80%	40 - 50	Poor

A combination of iron and manganese produced the best culture growth from all considerations. Both of these metals were essential for the metabolism of glucose by desirable bacterial cultures. The addition of zinc promoted a more filamentous sludge with an increased SVI. Iron and zinc alone produced a very filamentous microorganism growth. Iron alone supported growth, but was filamentous in nature. The addition of cobalt and copper markedly reduced the sludge flocculation and substrate utilization.

In general, iron and manganese are the only required and desired trace metals for glucose substrate. This produces a sludge with all of the desired characteristics. Addition of zinc promotes the growth of fungi with a poor settling sludge resulting. Cobalt and copper inhibit the substrate utilization and disrupt the flocculating characteristic of the sludge resulting in poor efficiencies. These solids do not settle and remain in the effluent. This produces a high effluent BOD plus a reduction in the mixed liquor solids concentration.

A qualitative summary of the mixed culture growth on the acetate substrate is given in Table 21. Again, iron and manganese were the two required trace metals. Acetate utilization was very good with iron or manganese alone, or a combination of the two. Manganese tended toward a higher SVI. This was generally 100 or less and would not be considered as a true

filamentous growth. The addition of zinc with iron and/or manganese tended also to maintain a higher SVI than iron alone. However, this effect was not great and may have little significance.

Cobalt and copper completely disrupted the flocculating characteristics of the sludges. Large quantities of dispersed cells were in the effluents markedly reducing the treatment efficiency. As long as iron was present, substrate utilization was good when the substrate contained copper and cobalt. Elimination of the iron produced failure of the systems as the substrate utilization was only 75 to 85 percent. With the accompanying dispersed cells, the systems loose the mixed liquor solids and ultimately fail.

TABLE 21

MIXED CULTURE GROWTH ON ACETATE SUBSTRATE

Metals Added	Growth	Substrate Utilization	SVI	Sludge Flocculation
Fe	Excellent	98%	50 - 70	Excellent
None	Very Poor	55 - 60 %	60 - 70	Very Poor
Fe, Mn	Excellent	97%	70 - 100	Excellent
Mn	Excellent	98%	100	Excellent
Fe, Mn, Zn	Excellent	98%	60 - 100	Excellent
Mn, Zn	Excellent	98%	70 - 90	Excellent
Fe, Mn, Zn, Co	Poor	97%	55 - 70	Poor
Mn, Zn, Co	Poor	85%	20 - 40	Very Poor
Fe, Mn, Zn, Co, Cu	Poor	97%	50 - 65	Poor
Mn, Zn, Co, Cu	Poor	75%	40 - 50	Very Poor

As with glucose, iron and manganese are the only required trace metals. The addition of zinc does not improve or impair the efficiency of the process. Copper and cobalt are undesirable because of the effect these metals have on the growth characteristics of the cultures.

It must be remembered that these metals are only active in the biological systems when they are soluble. Chemical environments which either render the metals totally insoluble or reduce the solubility below

some critical level will change the response of the microbial cultures to the presence of these metals. Addition of these metals to the substrate does not always mean they will be soluble and active in the growth of the organisms. Additions of as much as 25 mg/l of iron to substrate used in biological systems has resulted in iron concentrations of 0.1 to 0.2 mg/l. The remaining iron was precipitated by hydrolytic reactions and phosphate interaction. Extreme care must be exercised in analysing the growth response of microorganisms to the absence or presence of various metals.

CONCLUSIONS

The following conclusions are drawn from the results of this study:

1. Extreme care must be exercised in the preparation of chemically defined media if precipitation of trace metals is to be prevented.
2. The only trace metal required for growth of the fungus, Fusarium , on acetate, glucose and glycerol media was iron.
3. Zinc was a stimulant for Fusarium growth on acetate and glucose, but was not required for moderate growth.
4. The only trace metal required for growth of the fungus, Geotrichum on glucose and glycerol media was zinc. This organism required only iron for growth on acetate medium.
5. Iron was a stimulant for Geotrichum growth on glucose and glycerol, but was not required for moderate growth.
6. Glucose media containing only the trace metals iron and manganese yielded the optimum growth characteristics for the mixed culture studies.
7. Acetate media containing only the trace metals iron or manganese yielded the optimum growth characteristics for the mixed culture studies.

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